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(54) **Title:** ENGINEERED LIVING TISSUE SUBSTITUTE

(57) **Abstract:** The present invention relates generally to the field of tissue engineering and in particular to the production of tissue films or cell matrices, which can be used as a living tissue substitute or an artificial tissue construct in tissue repair or replacement.

Title

Engineered Living Tissue Substitute

Field of the Invention

The present invention relates generally to the field of tissue engineering and in particular to the production of tissue films, sheets or cell matrices, which can be used as a living tissue substitute or an artificial tissue construct in tissue repair or replacement.

Background to the Invention

The goal of tissue engineering is to repair or replace tissues and organs with artificial tissue constructs. Scaffolds are typically used for this process, giving mechanical support, and assisting in cell migration and attachment, cell retention and delivery at the site of repair. The scaffold thus mimics the natural matrix found in the body. In other situations, films or layers of tissue itself may be suitable for the repair or transplant. The cells in such films should thus be as similar as possible to those produced naturally by the body. It is thus important that cells grown into these tissue layers or films are properly aligned, and metabolise to produce factors that the tissue they are destined to replace would produce.

The extracellular matrix is a complex variety of glycoproteins and proteoglycans, which provides tissue integrity, acts as a native scaffold for cell attachment and interaction and acts as a reservoir for growth factors. For most connective tissues, collagen makes up the bulk of the extracellular matrix, where it functions as a structural protein as well as a binding partner for glycans that store growth factors.

Collagen is a family of extracellular matrix proteins, the most abundant being type I found in skin, tendon, bone, corneal, type IV found in all basement membranes and type VII found in the basement membrane of skin, oral mucosa and cornea. These collagen assemblies differ depending on the tissue location and function.

The deposition of a collagen matrix depends on the conversion of denovo synthesised pro-collagen to collagen in the extracellular space immediately before its release into the space. This limiting step for collagen matrix deposition is very slow *in vitro*, both in monolayer cultures and in three dimensional scaffolds.

For a number of tissue engineering applications, tissue grafts (autografts, allografts or xenografts) are considered to be the 'gold standards'. However, the limited supply of autografts and certain donor site morbidity restricts their utilisation. The use of

allografts and xenografts has also been questioned due to poor success rates, possibilities of immune rejection and potential transmission of disease. To this end, tissue engineering was pioneered as the only viable alternative to the transplantation crisis. Several degradable and non-degradable synthetic materials have been evaluated over the years [2-10]. However, non-degradable synthetic materials may become harmful due to mechanical impingement or infection and require a second operation, whilst the degradation products of biodegradable synthetic materials could be deleterious to the surrounding cells and tissues. Natural biomaterials such as collagen [15-18], gelatin [19, 20] and fibrin [21] have been used as raw materials for scaffold fabrication with promising early results. In particular, the use of collagen as a raw material for scaffold fabrication has been advocated because, as a natural occurring biopolymer that constitutes approximately one third of the total body proteins, it is perceived by the body as a normal constituent rather than foreign matter . Despite advancements in purification methods and analytical assays that have assured low immunogenicity and antigenicity, collagen remains an animal derived by-product and its use in clinical applications can be limited due to concerns of inter-species transmission of disease, especially for collagen extracted from bovine tissues (e.g. Bovine Spongiform Encephalopathy and Creutzfeldt Jakob Disease). In fact, 2-3% of patients have an immune response to collagen implants using collagen derived from land-based animals. For this reason, human recombinant collagen has been investigated for scaffold fabrication. Although several expression systems have successfully produced human recombinant procollagens, in all cases procollagen expression levels have been low (15mg/ml for mammalian cell culture; 15mg/ml in yeast; and 60mg/ml in baculovirus) which was prohibited commercialisation and clinical applications. Moreover, whilst recombinant collagens can be expressed in a thermo-stable triple helical form, they lack specific domains otherwise present in native fibrillar collagens, which can compromise their biological function and further reduce their use. In light of this, it has been predicted that companies developing new implantable products are more likely to focus on human collagen products, rather than on products utilising animal-sourced collagen.

Indeed, advancements in molecular and cell biology have allowed the use of cell-based therapies for tissue engineering and regenerative medicine applications. The concept is

that replacement, repair and restoration of function can be accomplished best using cells that will create their own host-specific extracellular matrix. Indeed, cells are *professional matrix makers* and assemble into large aggregates together with ligands, growth factors and other matrix components with a precision and stoichiometric efficiency that is still unmatched by man-made devices, recombinant technology-derived components or chemical compounds. Cell-based injectable systems and cell-sheets derived from autologous primary cell isolates; from established cell lines; and from a variety of stem cells have been used for numerous clinical targets, including cornea, skin, blood vessel, cartilage, lung, cardiac patch, oesophagus and periodontal applications.

Cultured cells deposit extracellular matrix (ECM) molecules and form cell-to-cell junctions. However, typical proteolytic harvest (by trypsin) digests both deposited ECM and cell-to-cell junctions. In contrast, culture dishes covered with a temperature-responsive polymer allow harvesting of intact cell sheets along with their deposited ECM, by simple temperature reduction. Despite the success of cell sheet tissue engineering in regenerative medicine, this technology has still not taken off primarily due to the substantial long period of time required to culture the cells and develop an implantable cell-sheet.

Herein, for first time, we describe the production of cell-sheets within 24-48h from human cells using a biophysical approach that governs the intra- and extra-cellular milieu in multicellular organisms, termed macromolecular crowding, that invites cells to create their own matrices. The principal that the approach is based on is that the deposition of a collagen matrix depends on the conversion of *de novo* synthesised procollagen to collagen in the crowded extracellular space or immediately before its release into the same. The rate limiting step of collagen I deposition is the proteolytic conversion of procollagen to collagen. This step is catalysed by procollagen *C*-proteinase and proteolytic modification of its allosteric regulator. *In vivo,* the extracellular space is highly crowded (Figure 1); even dilute body fluids are highly crowded: blood contains 80g/l protein; urine contains 36-50g/l solids, and the conversion of procollagen to collagen takes place very fast (Figure 2). However, *in vitro* cells are grown in highly dilute conditions; this, in the human body, would represent a medical pathology. However, this situation can be remedied, by adding macromolecules

of defined hydrodynamic radius to culture media and thus creating excluded volume effects with defined volume fraction occupancies. Conventional cell culture systems are far from crowded environments (Table 1) and in this dilute, far from physiological, environment the deposition of matrix is very tardy.

Table 1: Concentration of solids in conventional cell culture system

MEDIA	COMPANY	SOLID CONCENTRATION
Minimum Essential Medium	Invitrogen, Cat.No: 10370021, 10370039,10370047,10370054,10370070	11.52g/l
F12 Nutrient Mixture	Invitrogen, Cat. No: 21765029	11.87g/l
RPMI 1640 Medium	Invitrogen, Cat. No. 11835030, 11835055,11835063, 11835071	12.39g/l
Ham F10 Nutrient Mixture	Invitrogen, Cat. No. 22390017, 22390025	16.55g/l
DMEM: F12 Medium	ATCC, Cat. No. 30-2006	16.78g/l
DMEM High Glucose (4.5g/) and L-glutamine	Invitrogen, Cat. No.41965039, 41965047	17.22g/l

In an attempt to modulate the *in vitro* micro-environment and closely emulate the *in vivo* setup, macromolecules have been used to crowd the culture media (Table 2). Under crowded conditions, thermodynamic activities increase by several orders of magnitude and biological processes, such as enzymatic activities and protein folding can be dramatically accelerated.

Table 2: Macromolecules that have been used to-date as crowding agents

CROWDER USED	RESULTS	REFERENCE
Sucrose and glucose (monomers for dextran and Ficoll™), PEG 0.2 Kda Ficoll™ 70Kda, Dextran T70Kda, PEG 8Kda, PEG 35KDa	Ineffective MMC significantly increased enzymatic activity	Zimmermann and Harrison, 1987, PNAS, 84: 1871-1875
Ficoll™ 70KDa, Dextran 70KDa	Faster protein folding rates	van den Berg *et al.*, 1999, EMBO Journal, 18(24): 6927-6933
PEG 3.5Kda, Ficoll™ 70KDa	MMC dramatically increase fibrillation of unfolded proteins	Munishkina *et al*, 2008 Biochemistry, 47(34): 8993-9006
Ficoll™ 70Kda, Dextran 70KDa	MMC dramatically accelerated the nucleation step of fibril formation of human Tau fragment & prion protein	Zhou *et al.*, 2009, Journal of Biological Chemistry, 284(44):30148-30158
Dextran Sulphate 500 Kda (negative; 46nm radius) PSS 200Kda (negative :22nm radius) Ficoll™ 400Kda (neutral; 4.5nm radius) Ficoll™70Kda (neutral; 3nm radius)	Dramatically accelerated extracellular matrix composition	Lareu *et al.*, 2007 FEBS letters, 581 : 2709-2714 Lareu *et al.*, 2007 BBRC, 363: 171-177 Lareu *et al.*, 2007 Tissue Engineering., 13(2): 385-391

Animal extracted and recombinant collagen is used in several tissue engineering applications since under appropriate conditions of temperature; pH; and ionic strength since they will self-assemble to produce collagen fibres indistinguishable from fibres found *in vivo.* However, animal extracted collagens are responsible for immune response, whilst recombinant collagen is not biologically active since it is not produced with the appropriate post-translation modifications.

Lareu *et al* (2007) describe a dramatic enhancement in collagen matrix deposition by using large negatively charged polameric macromolecules to create an excluded volume effect in long fibroblast cultures. Chen *et al* (2004) similar results were achieved using a cocktail of macromolecules using dextran sulphate and Ficoll molecules. Again the crowders are negatively charged molecules with large hydrodynamic radii. Similarities have been shown with neutral and negatively charged dextran sulphate by Lareu (2007) and Peng and Raghunath.

[In press]

The present inventors have surprisingly found that by using poly-dispersed macromolecular crowders, that cell metabolism and extracellular matrix production can be enhanced to such a level that significant quantities of tissue substitute films are produced after as little as 48 hours. It has never previously been shown that poly-dispersity of a macromolecule is key to enhancing the production of tissue substitutes. This knowledge allows the identification of molecules which are suitable for this purpose and the mixing of molecules to enhance the polydispersity of a mixture of molecules.

Object of the Invention

It is thus an object of the present invention to provide a process for the production of tissue substitutes or artificial tissue constructs which is rapid. These tissue substitutes or constructs can take the form of tissue layers or sheets of cells. In particular the object of the invention is to provide a method of producing commercially viable quantities of tissue substitute films within a period of 2-5 days. In particular it is an object of the invention that the substitute can be produced within in about 48 hours.

Another object is to address the shortfalls of animal-extracted and recombinant molecules and to provide a new method for producing tissue substitutes without the addition of any animal compounds. Such a product would harbour no risks for interspecies transmission of disease (as compared to animal extracted collagen) and also is fully biologically active (as opposed to recombinant collagen) since it is produced *naturally* from cells.

The presence of macromolecules in the culture media will closely emulate the *in vivo* setup and facilitate the fast deposition of extracellular matrix as typified by collagen type I (Figure 3) or fibronectin production. It is an object to apply the principles of macromolecular crowding on human fibroblast cultures are applied to produce, isolate and purify for first time a human tissue substitute that can be used as raw material for biomedical devices or in tissue repair or regeneration. This tissue produced will have advantages over recombinant technologies since it will be fully biologically functioning (produced fromhuman cells) and will avoid therisk of interspecies transmission of disease, since no animal serum is used. Moreover, this system can

facilitate rapid production of host-specific tissue substitutes from the patient's own cells and as such will avoid even immune rejection problems from implantation of materials from another subject.

The presence of macromolecules in the culture media will closely emulate the *in vivo* setup and facilitate the fast deposition of collagen type I (Figure 4). Moreover, it is an object that other extracellular matrix molecules will be deposited (e.g. fibronectin). *In vitro* studies have demonstrated that fibronectin deposition is necessary for the formation of collagen fibrils to occur in the ECM.

An object is to employ the principles of macromolecular crowding on human cells and produce live human cell sheets for tissue engineering and regenerative medicine applications. This system will facilitate production of host-specific cell-based scaffolds from the patient's own cells and as such will avoid immune rejection problems from implantation of materials from another subject. In addition, this system can also be used to produce host specific proteins (e.g. collagen) that can be used for tissue engineering applications. Moreover, this system can be used to accelerate in vitro biological processes (e.g. enzyme activity; degradation of proteins, etc).

The tissues so produced may be used for tissue engineering applications. Such applications include Tendon regeneration, Bone regeneration, Nerve regeneration, Cornea regeneration, Skin regeneration etc, Drug delivery, drug discovery, gene discovery, In vitro systems (e.g. development of cancer therapeutics; development blood-brain barried systems, etc) Gene delivery, coating of medical devices to avoid immune response, and tissue glues/adhesives.

Such products will replace products that are based on animal extracted molecules and products that are based on human recombinant molecules.

Summary of the Invention

According to the present invention there is provided a method for the production of a tissue substitute comprising culturing cells in the presence of one or more macromolecular crowders, wherein the macromolecular crowders are large poly-dispersed macromolecules. Two or more macromulecular crowders may be preferred. The macromolecules may be negatively charged or neutral macromolecules.

The large poly-dispersed macromolecules may be selected from the group comprising carrageenan, dextrans, dextran sulphate, Ficoll™, bovine serum albumin, sodium alginate, polyethylene glycol, sepharose-CL and polysodium-l-styrene sulphonate. Particularly preferred is carrageenan. Carageenan may be used in combination with one or more of the molecules as defined above.

The invention may comprise a method of producing a tissue substitute comprising culturing cells in the presence of a large macromolecular crowder with the proviso that the crowder is not dextran sulphate alone. In other words, dextran sulphate is used with another crowder.

Carrageenans are a family of linear sulphated polysaccharides extracted from red seaweeds. There are several types of carrageenan:- Kappa, lambda and iota, all of which would be suitable for use in this invention. Particularly preferred is kappa carageenan. Also preferred is lambda carrageenan. The macromolecular crowder may be used at between 75 μg/ml and 37.5 mg/ml, with the amount used depending on the size of the crowder.

The cells may be selected from lung fibroblasts, skin fibroblasts, corneal fibroblasts, dermal fibroblasts, human umbilical artery smooth muscle cells, human tenocytes and normal human osteoblasts.

The cells may be cultured in the presence of culture medium supplemented with fetal bovine serum, human serum, ascorbic acid phosphate, or a combination thereof. The human serum may be used at 0.1% to 40% volume to volume. Suitable concentrations of serum include, 0.5% to 30%, and 5% to 20%.

The invention also provides a cell matrix film, tissue sheet or tissue substitute whenever produced by a method as described above.

As used herein the term "molecules" includes molecules, spheres, particles and polymers. Table 2 indicates the radius for a number of molecules, but should not be taken to imply that all of the molecules of the invention are spheres. As used in here the term "poly-dispersed" means that the molecules have a broad range of size, shape and mass characteristics, as opposed to molecules which have a uniform size, shape and mass distribution which are mono-dispersed molecules. Polymer materials are poly-dispersed if their chain length varies over a wide range of molecular masses. This poly-dispersity can be seen in Figure 11A-E. A number of neutral and negatively charged

macromolecules are poly-dispersed. It is however apparent from the figure that carrageenan is the most poly-dispersed of the molecules tested in this invention, and the inventors hypothesis that this is the reason for the superiority of carrageenan amongst the molecules tested in the method of the present invention. It would however be possible to increase the polydispersity of the crowder, by using a combination of two or more crowders. For example, a mixture of carrageenan and dextran sulphate would be more poly-dispersed than carrageenan alone.

This invention enables the production of substantial amounts of cell tissue within only 48h, whilst the current *in vitro* systems take in excess of 6 weeks to produce the same amount of tissue.

To further eliminate animal products from the process, human serum was used and it surprisingly increased further the tissue and extracellular matrix production as typified by collagen production. A number of cell types were screened (e.g. lung fibroblasts, skin fibroblasts, smooth muscle cells) and surprisingly, it was found that smooth muscle cells can be very rapidly produced by the method of the invention. It was also found that poly-dispersed macromolecules occupy more space and facilitate higher collagen production in comparison to mono-dispersed molecules, indicating higher tissue layer production. Low serum concentration is sufficient for high collagen production which makes this invention even more financially viable. Ultimately, herein we describe for very first time the rapid production of human tissue substitutes that can be used for any tissue engineering applications.

Brief Description of the Drawings

Figure 1: Immunofluorescent images of skin and cornea tissue. Cells (stained blue with DAPI) are packed in the dense extracellular matrix.

Figure 2: *In vivo* collagen biosynthesis. Cells synthesise collagen with intact pro-peptide extensions. During, or following, secretion in the heavily crowded extracellular space, pro-peptide cleavage by specific proteinases takes place that triggers the spontaneous quarter staggered self-assembly of collagen molecules into fibrils. Subsequently, the native cross-linking pathway of lysyl-oxidase takes place that give rise to collagen fibres.

Figure 3: In conventional cell-culture system the collagen deposition is very slow due to the very dilute, far from physiological, conditions (left panel). However, under crowding conditions, the pro-peptide extensions are cleaved by the specific proteinases and collagen deposition takes place (right panel).

Figure 4: SDS-PAGE and complimentary densitometric analysis demonstrated that WI-38 fibroblasts at the presence of 500Kd DxS (100//g/ml) deposited highest amount of ECM after 2 days in culture and at FBS concentration between 0.5 and 1%. Standard: 100μg/ml bovine collagen type I (Symatese Biomateriaux, France).

Figure 5: (top) SDS-PAGE and complimentary densitometric analysis indicates that the 0.5% and 1% serum concentration facilitates maximum ECM deposition for WI38 and WS1 fibroblasts (/?<0.001) after 2 days in culture at the presence of 100μg/ml 500KDa dextran sulphate. SDS-PAGE analysis indicates the 0.5% serum concentration facilitates maximum collagen type I deposition for WI38 fibroblasts. For the WS1 fibroblasts no difference was observed in collagen deposition for 0.5%> and 1% serum concentration and both these concentration facilitated higher collagen deposition than any other serum concentration after 2 days in culture at the presence of dextran sulphate (bottom) Complementary densitometric analysis confirms the high deposition of collagen type I under crowded conditions at 0.5%> serum concentration for both WI38 and WSl fibroblasts.

Figure 6: Immunocytochemistry experimentation demonstrates the high deposition of collagen type I and fibronectin after 2 days in WI38 culture, at 0.5%> and 1% serum, at the presence of dextran sulphate. Co-localisation of fibronectin and collagen type I is also apparent.

Figure 7: Immunocytochemistry experimentation demonstrates the high deposition of collagen type I and fibronectin after 2 days in WS1 culture, at 0.5% serum, at the presence of dextran sulphate. Co- localisation of fibronectin and collagen type I is also apparent.

Figure 8: SDS-PAGE analysis indicates the highest collagen deposition in WI38 and WS1 culture in the presence of carrageenan.

Figure 9: Immunocytochemistry experimentation corroborated the high collagen type I deposition at the presence of carrageenan in the culture of WI38 and WS1 fibroblasts after 2 days in culture.

Figure 10: The dynamic light scattering analysis demonstrated that the negatively charged macromolecules (carrageenan, dextran sulphate and PSS) had significant higher hydrodynamic diameter (Z-Ave, d.nm) than their neutral counterparts (Ficoll™ 70, Ficoll™400).

Figure 11A-E: Poly-dispensity levels of macromolecules: The size dispersion by intensity confirms that the negatively charged macromolecules (carrageenan, dextran sulphate and PSS) are highly polydispersed and among them, carrageenan is the most polydispersed.

Figure 12: The influence of macromolecular crowding in human umbilical smooth muscle cells in the presence of various concentrations of ascorbic acid.

Figure 13: SDS-PAGE and complementary densitometric analysis indicates that human serum induced higher ECM deposition in comparison to FBS counterparts.

Figure 14: Immunocytochemistry evaluation further corroborated the higher ECM deposition in the presence of human serum, when compared to FBS in the culture of WS1 and WI38 fibroblasts.

Figure 15: The morphology for WI38 and WS1 and smooth muscle cells was not affected independent of the serum concentration, the macromolecule; and the period in culture.

Figure 16: A Lamar BLUE (TM) results as a function of FBS concentration statistics.

Figure 17: A Lamar BLUE (TM) results as a function of FBS concentration statistics.

Figure 18: Complementary densitometric analysis shows that carrageenan induces maximum collagen deposition in WS1 and WI38 fibroblast culture.

Figure 19: Complementary densitometric analysis shows that human serum increased collagen deposition in WS1 and WI38 fibroblast culture when compared to FBS.

Figure 20: A structural analysis by atomic force microscopy of the tissue substitute for the invention.

Detailed Description of the Drawings

Materials and Methods

Cell culture:

Human lung fibroblasts (WI-38; American Tissue Culture Collection), Human skin fibroblasts (WS-1; American Tissue Culture Collection) were routinely cultured in

Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5%$C0_2$. Commercially available Smooth Muscle Growth Medium-2 (Clonetics® SmGM® -2) and supplements (SmGM-2 SingleQuots) were used for human umbilical arterial smooth muscle cells (HUASMC; Clonetics® UASMC-Human Umbilical Artery Smooth Muscle Cells; Lonza CC-2579). Cells were seeded at 50,000 cells/well in 24-well plates and were allowed to attach for 24 hours. After 24 hours the medium was changed with medium containing macromolecular crowders (100μg/ml dextran sulphate 500 kDa; 37.5mg/ml Ficoll™70 and 25mg/ml Ficoll™400; 100μg/ml Polysodium-4-Styrenesulfonate, 75μg/ml carrageenan and 100 μl/ml sepharose-CL) and with various percentages of FBS (0%, 0.5%, 1%, 2%, ***5%,*** and 10%). To induce collagen synthesis, fibroblasts were supplemented with 100 µM L-ascorbic acid phosphate. The HUASMC was supplemented with various concentration of ascorbic acid (0µM, 25µM, 50 µM and 100 µM). In some of the experiment commercially available human serum (Lonza, Belgium) was also used as supplement in place of FBS.

Phase contrast microscopy:

The influence of various crowders and FBS percentages on cell morphology was evaluated using phase-contrast microscopy at day 2, 4 and 6. Images of the cells were taken by inverted microscope (Leica microsystem, Germany) and images were analyzed with the help of LAS EZ 2.0.0 software.

Collagen extraction:

At the end of culture time points, culture media were collected into separate vials, whereas cell layers were washed twice with Hank's balanced salt solution (HBSS). Both culture medium and washed cell layer were digested with porcine gastric mucosa pepsin in a final concentration of lmg/ml in 0.5M acetic acid. Samples were incubated at 37°C for 2 hours with gentle shaking followed by neutralization with 0.1 N NaOH.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

Cell layer and Medium samples were analyzed by SDS-PAGE under nonreducing conditions with Mini-Protean 3 (Bio-Rad Laboratories). 100µg/ml of bovine collagen type I (Symatese biomateriaux, France) was used as standards with every gel. Protein bands were stained with the SilverQuest® kit (Invitrogen) according to the manufacturer's protocol. Densitometric analysis of gels was performed with the help of

GeneTools analysis software (Syngene). Collagen bands were quantified by defining each band with the rectangular tool with background subtraction.

Immunocytochemistry:

Fibroblasts were seeded on 4-well Lab-Tek™ II chamber slides at 50,000 cells/chamber and after 24 hours of seeding cells were treated with crowders. After 2 days of culture, medium was removed and cell layers were washed with HBSS and fixed with 2% paraformaldehyde at room temparature for 15 min. After several washes in phosphate-buffered saline (PBS), nonspecific sites were blocked with 3% bovine serum albumin in PBS for 30 min. The cells were incubated for 90 min at room temperature simultaneously with Collagen I (Rabbit anti-human): dilution 1:100 and Fibronectin (Mouse anti-human) dilution 1:200 for 90 min. Bound antibodies were visualized using AlexaFluor®488 chicken anti-rabbit and AlexaFluor®555 goat anti-mouse 1:400 in PBS for 30min. Post-fixation was with 2% PFA for 15 min. Cell nuclei were counterstained with 4,6-diamidino- 2-phenylindole (DAPI) and slides were mounted with Vectashield® mounting media. Images were captured with an Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Dynamic light scattering (DLS) measurements:

Dynamic light scattering (DLS) measurements of macromolecules were done using Zetasizer Nano ZS90 (Malvern Instruments) at 25°C. Molecules were dissolved in HBSS, pH 7.4 for size (Z-Ave; d.nm) measurements, and for zeta (ζ)- potential measurements, macromolecules were dissolved in water. The measurement of zeta (ζ)-potential and size (Z-Ave. diameter in nano meter) were analysed by the help of Zetasizer software 6.12 (Malvern Instruments).

Cell metabolic activity (alamarBlue®):

alamarBlue® assay was performed to quantify the influence of various crowders and serum on metabolic activity of the fibroblasts. For HUASMC, apart from influence of crowders, effect of ascorbic acid supplementations (OμM, 25μM, 50 μM and 100 μM) on celfs metabolic activity was also analysed. Briefly, at the end of culture time points, cells were washed with HBSS and then diluted alamarBlue® was added. After 4 hours of incubation at 37°C, absorbance was measured at 550 and 595nm. Cell viability was expressed in terms of reduction percentage of alamarBlue®.

Results

A. Evaluation of different cells to identify the most suitable cell type for maximum collagen production.

Human lung fibroblasts (WI-38; American tissue culture collection), human dermal fibroblasts (WS-1; American tissue culture collection), and human umbilical arterial smooth muscle cells (huasmc; clonetics, UASMC-human umbilical arterial smooth muscle cells; Lonza cc-2579) were evaluated. Cells from pathophysiologies (eg hypertrophic scar) have not been evaluated since disease transmission may occur. Although we would have expected fibroblasts to produce more collagen, our data indicated that HUASMC deposited higher amounts of collagen- 1 when crowded with 10(^g/ml of DxS and 75µg/ml carrageenan.

B. Evaluation of different serum concentrations to identify the most suitable concentration for maximum collagen production.

To evaluate the optimum serum supply for maximum collagen type I deposition, first we tested 500kDa dextran sulphate (DxS) (100μg/ml) as a macromolecular crowder. We identified the 0.5% fetal bovine serum FBS supplementation as the optimal serum concentration for maximum collagen type I deposition. Our data indicate that WI-38 fibroblasts deposited higher extracellular matrix (ECM) in the presence of 500KDa DxS (100μg/ml) after 2 days in culture at FBS concentration ranging from 0.5 to 1.0% (/?<0.001). Having identified that maximum ECM deposition is achieved by day 2, we tested the influence of various FBS concentrations (0, 0.5, 1.0, 2.0, 5.0 and 10.0%) on the culture of WS1 fibroblasts. Again, we identified the 0.5% to 1% FBS supplementation as the optimal serum concentration for maximum ECM deposition.

C. Evaluation of different macromolecules, in regards to hydrodynamic radius, to identify the most suitable hydrodynamic radius for maximum collagen production.

After we identified the optimal serum concentration (0.5% FBS), we tested other crowding molecules (37.5mg/ml Ficoll™ 70 and 25mg/ml Ficoll™ 400; 100μg/ml polysodium-4-styrene sulfonate (PSS), 100μl/ml sepharose-CL and 75μg/ml carrageenan) cells that are customarily used for tissue engineering applications of lung, skin, lung and blood vessel. Our data indicate that macromolecular crowding significantly increased ECM deposition (/?<0.001) and among the macromolecules used, carrageenan induced the highest ECM deposition (/?<0.001). Carrageenan enhanced

collagen-I deposition up to 20-30 times more within 48 hours.

D. Evaluation of different concentrations of human serum to identify the most suitable concentration for maximum host-specific collagen production.

To eliminate any chance of interspecies disease transmission and xenotransplant rejection; we also tested commercially available human serum as an alternative to FBS. Surprisingly, 0.5% of human serum supply not only worked consistently for macromolecular crowding, it approximately doubled the deposition of collagen type I in comparison to 0.5% FBS supplementation.

E. Evaluation of deposition of other extracellular matrix molecules.

Apart from collagen type I, the fibronectin deposition was also analysed immunocytochemically to evaluate its pattern of deposition with collagen type I. Immunocytochemistry results further confirmed the enhanced deposition of collagen I and its co-localisation with fibronectin in the presence of macromolecular crowders as shown in the immunocytochemistry figures.

F. Evaluation of crowders on cell morphology and metabolic activity.

Cell morphology and viability were analysed using phase contrast microscopy and AlamarBlue® assay respectively at day 2, 4 and 6. Phase contrast microscopy revealed that the fibroblasts maintained their spindle-shaped morphology independent of the macromolecular crowder present or the serum concentration up to 6-days in culture. AlamarBlue® analysis demonstrated that cell metabolic activity was not affected, independent of the macromolecular crowder present or the serum concentration even up to 6- days in culture (/?>0.05).

G. Dynamic Light Scattering

The dynamic light scattering analysis demonstrated that the negatively charged macromolecules (carrageenan, dextran sulphate and PSS) had significant higher hydrodynamic diameter (Z-Ave, d.nm) than their neutral counterparts (Ficoll™ 70, Ficoll™400).

The size dispersion by intensity confirms that the negatively charged macromolecules (carrageenan, dextran sulphate and PSS) are highly polydispersed and among them, carrageenan is the most polydispersed.

Having identified carrageenan as promising new crowding molecule, we evaluated the influence of macromolecular crowding in human umbilical smooth muscle cells in the

presence of various concentrations of ascorbic acid (ascorbic acid was used to induce collagen production). Our data indicate the macromolecular crowding significantly increased ECM deposition (/?<0.001). However, the presence of ascorbic acid did not affected ECM deposition (/?>0.05).

Conclusion

Figure 20 shows a preliminary structural analysis by atomic microscopy of the human tissue substitute of the invention. Tissue substitute shows a quartered staggered arrangement in which collagen fibres are aligned and similar to those found in native tissue. Thus the tissue matrix or substitute of the invention imitates native tissue.

Thus taking collagen production as a measure of metabolic activity and the stability of the artificial tissue construct, the invention has shown that the growth of cells in a macro molecular crowded environment using highly polydispersed molecules results in an appropriately aligned and functionally metabolising cell layer.

In particular, macromolecular crowding enhances the extracellular matrix (ECM) deposition from human lung, skin, bone and tendin cells. It also enhances ECM deposition even at low serum concentrations. Macromolecular polydispersity ehances ECM deposition. Most importantly, macromolecular crowding des not affect cell morphology, metabolic activity and viability.

The words "comprises/comprising" and the words "having/including" when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination.

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Claims

1. A method for the production of a tissue substitute comprising culturing cells in the presence of one or more macromolecular crowders, wherein the macromolecular crowders are large poly-dispersed macromolecules.

2. A method as claimed in claim 1 wherein the macromolecules are neutral or negatively charged macromolecules.

3. A method as claimed in claim 1 or claim 2 wherein the large poly-dispersed macromolecules are selected from the group comprising carrageenan, dextrans, dextran sulphate, Ficoll™, bovine serum albumin, sodium algerate, polyethylene glycol, sepharose-CL and polysodium-l-styrene sulphonate.

4. A method as claimed in claim 3 wherein the macromolecule is carrageenan.

5. A method as claimed in any preceding claim wherein the cells are selected from lung fibroblasts, dermal fibroblasts, skin fibroblasts, human terocytes, normal human osteoblasts and human umbilical artery smooth muscle cells.

6. A method as claimed in claim 5 wherein the cells are human umbilical smooth muscle cells.

7. A method as claimed in any preceding claim wherein the cells are cultured in the presence of culture medium supplemented with fetal bovine serum, human serum, ascorbic acid phosphate, or a combination thereof.

8. A method as claimed in claim 7 wherein the cells are supplemented with human serum.

9. A method as claimed in any claim 8 when the human serum is used at 0.5% to 1% volume to volume.

10. A method for the production of a tissue substitute substantially as described herein with reference to the Examples or accompanying drawings.

11. A tissue substitute whenever produced by a method as claimed in any preceding claim.

12. A tissue substitute as claimed in claim 11 which is selected from corneal tissue, skin tissue, tendon tissue, bone tissue, lung tissue and muscle tissue.

13. A method of increasing the speed of an *in vitro* assay comprising adding a large macromolecular crowder to the assay reagents.

14. A diagnostic comprising a large macromolecular crowder which is a large poly-dispersed macromolecules.

15. A cell culture medium containing a large macromolecular crowder.

Figure 1:

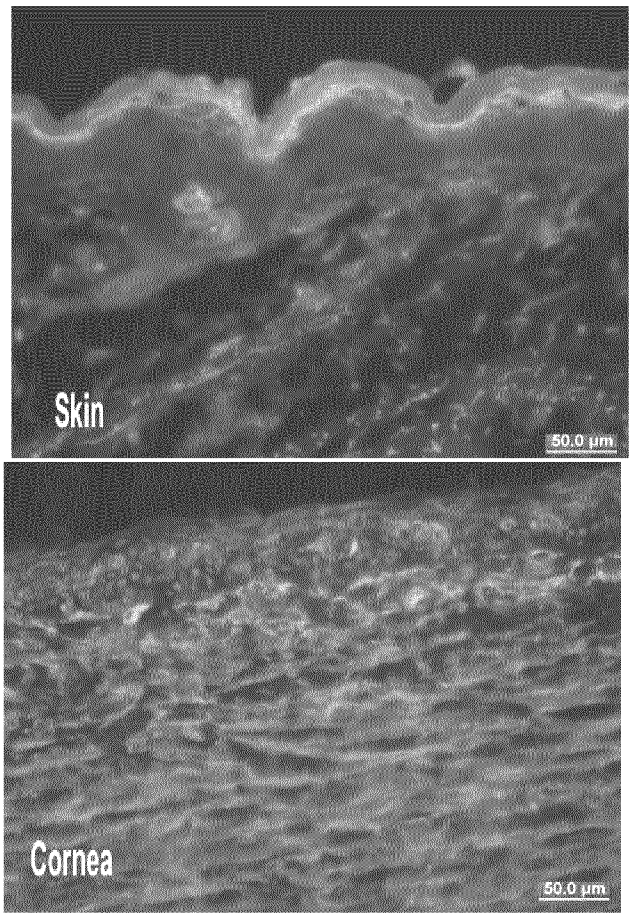


Figure 2:

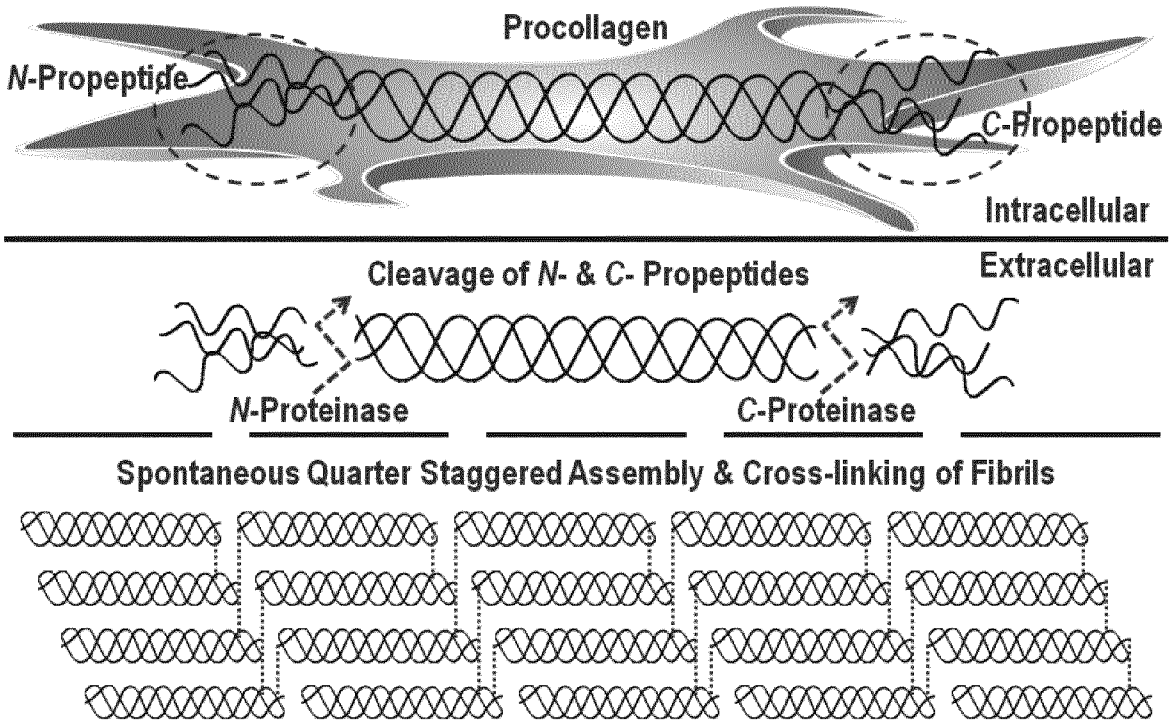
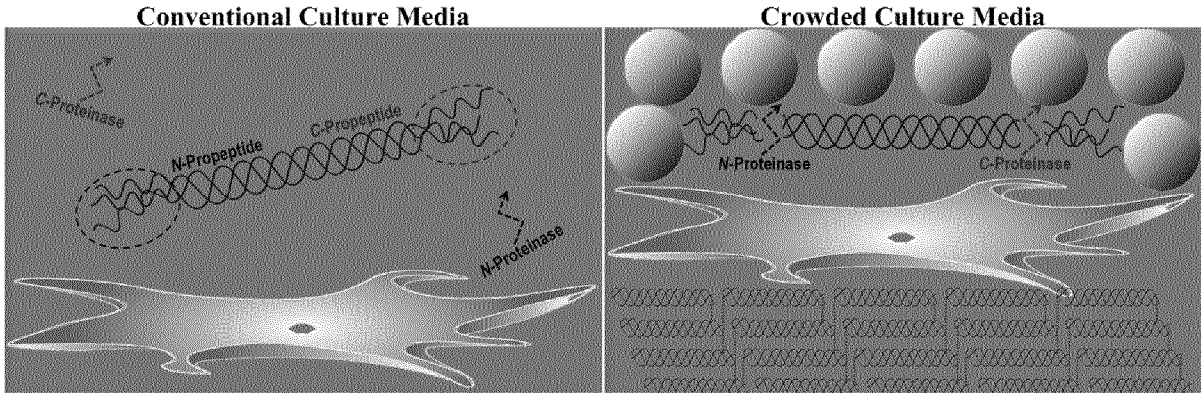


Figure 3:



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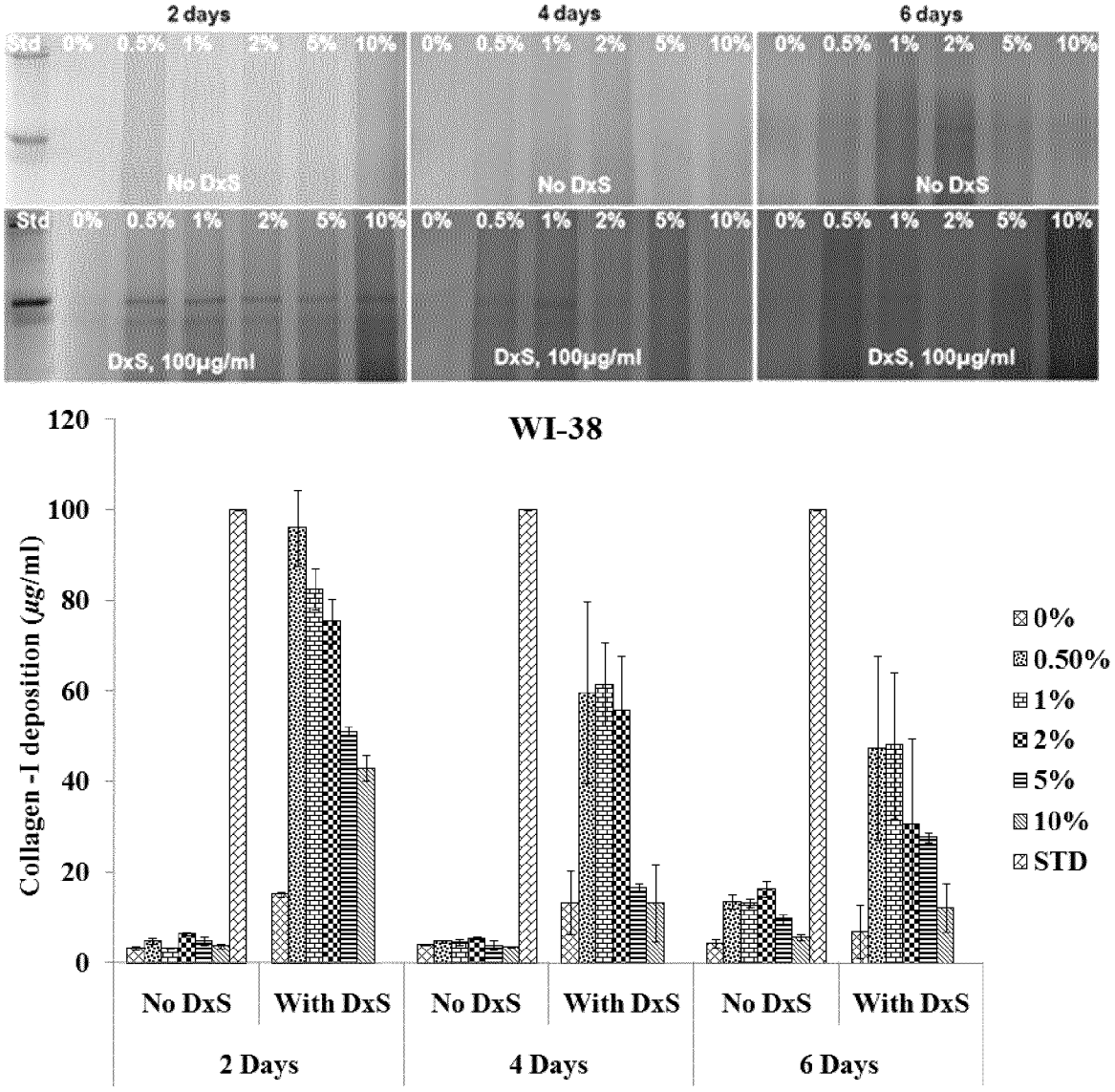


Figure 5:.

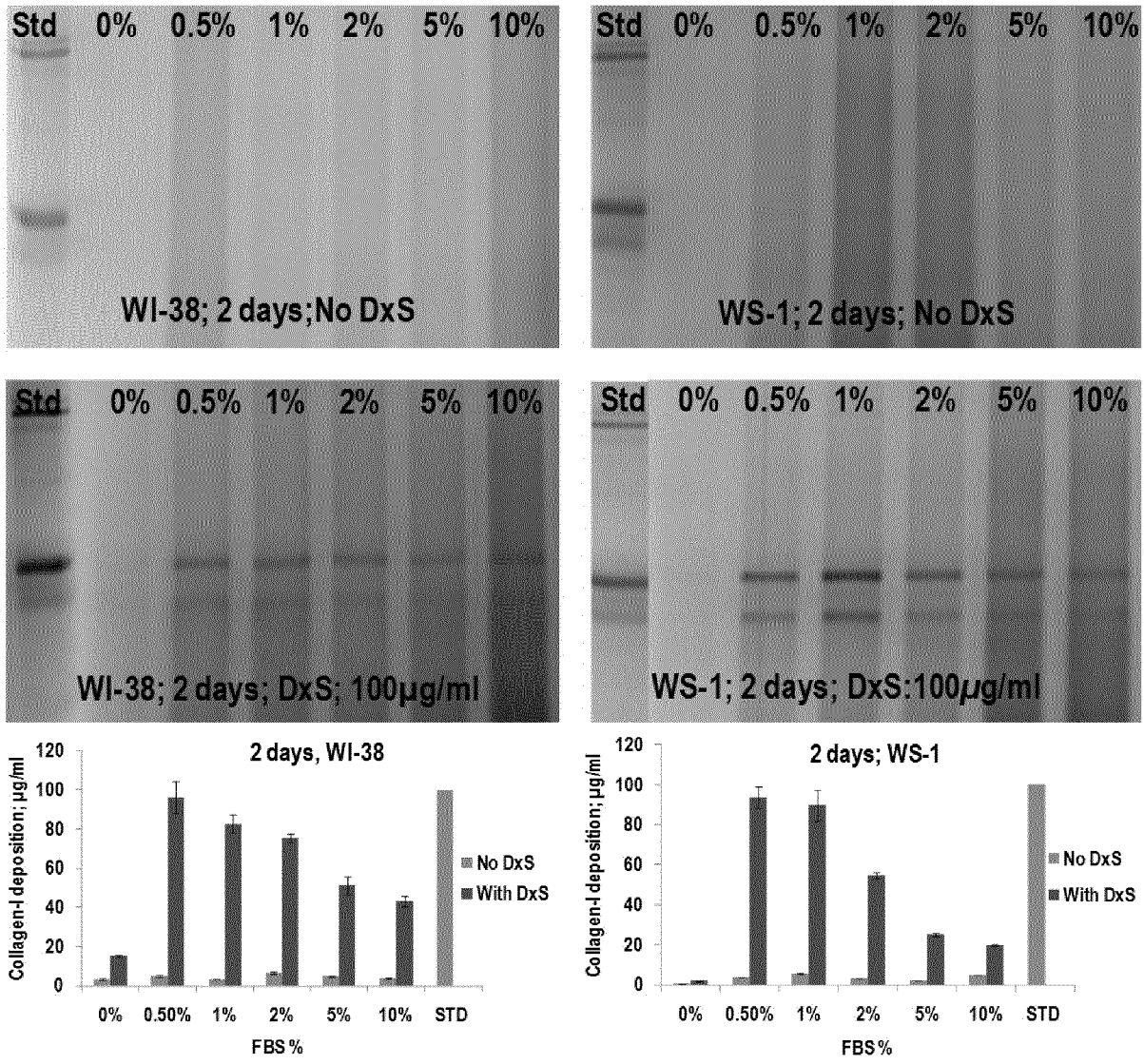
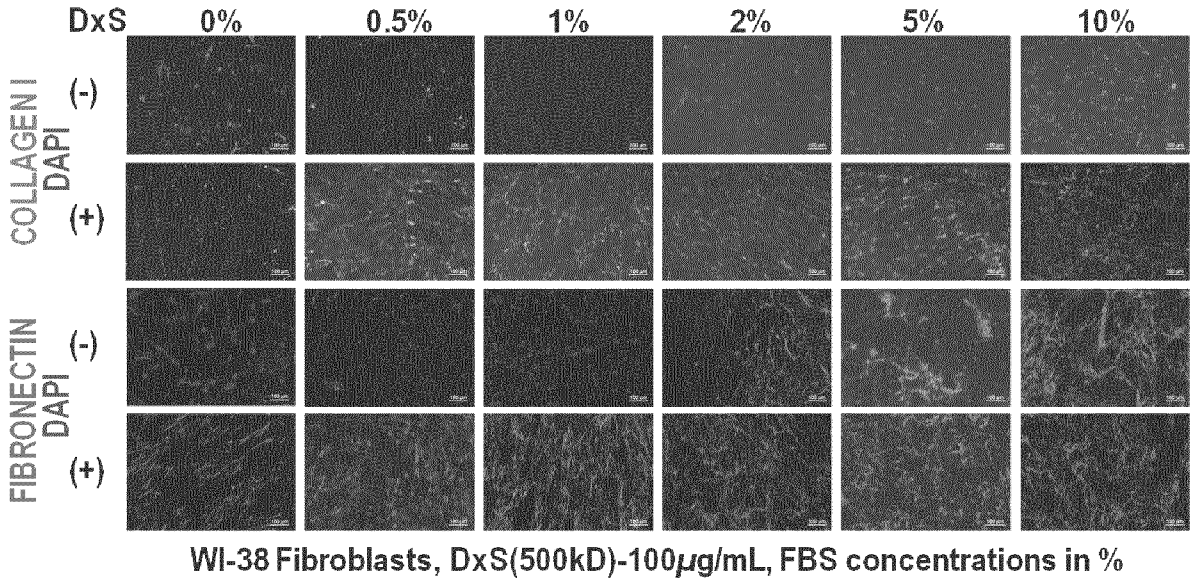

Std 0% 0.5% 1% 2% 5% 10%
WI-38; 2 days;No DxS
Std 0% 0.5% 1% 2% 5% 10%
WS-1; 2 days; No DxS
Std 0% 0.5% 1% 2% 5% 10%
WI-38; 2 days; DxS; 100µg/ml
Std 0% 0.5% 1% 2% 5% 10%
WS-1; 2 days; DxS:100µg/ml
2 days, WI-38
Collagen-I deposition; µg/ml
120
100
80
60
40
20
0
No DxS
With DxS
0% 0.50% 1% 2% 5% 10% STD
FBS %
2 days; WS-1
Collagen-I deposition; µg/ml
120
100
80
60
40
20
0
No DxS
With DxS
0% 0.50% 1% 2% 5% 10% STD
FBS %

Figure 6:



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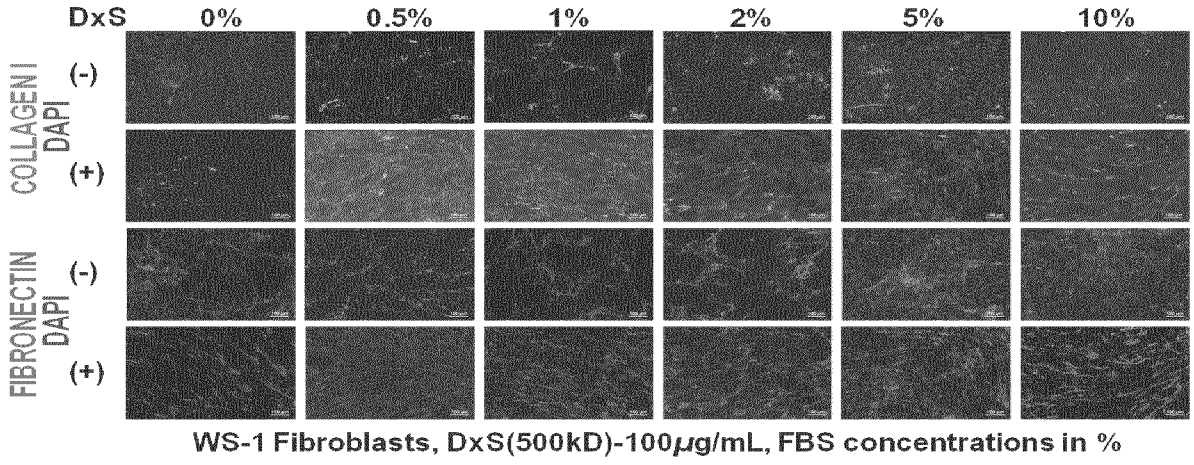

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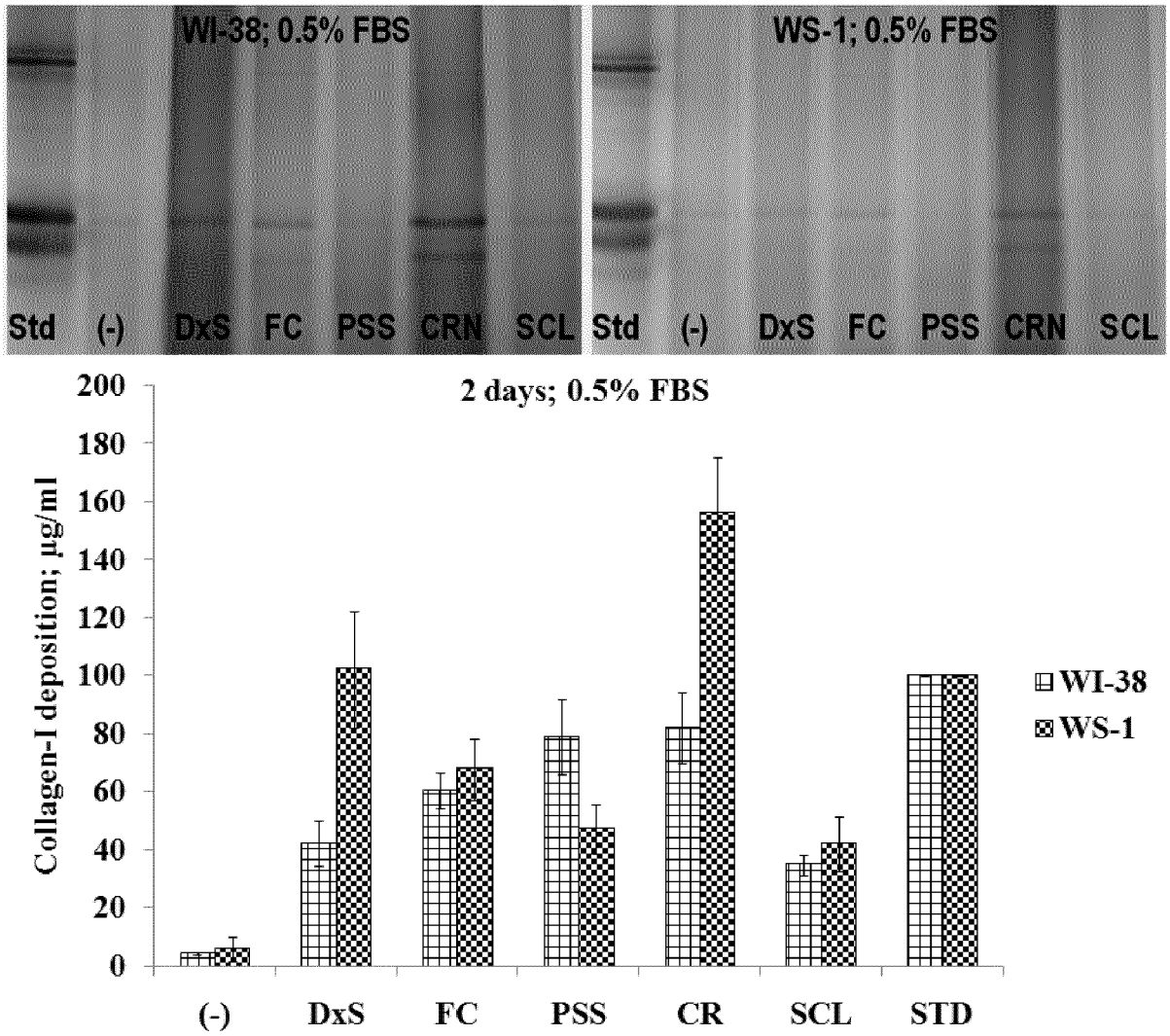

WI-38; 0.5% FBS
WS-1; 0.5% FBS
Std
(-)
DxS
FC
PSS
CRN
SCL
2 days; 0.5% FBS
Collagen-I deposition; µg/ml
200
180
160
140
120
100
80
60
40
20
0
(-)
DxS
FC
PSS
CR
SCL
STD
WI-38
WS-1

Figure 9:

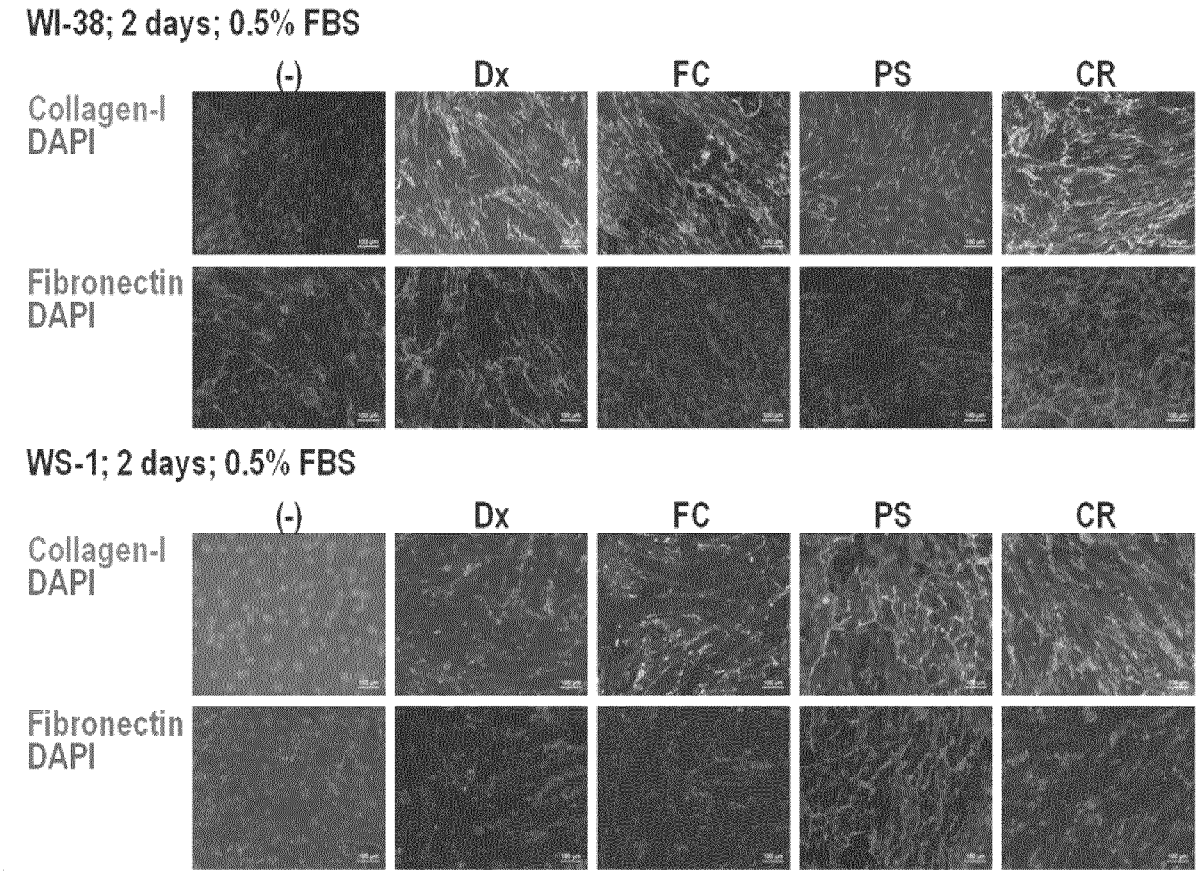


Figure 10:

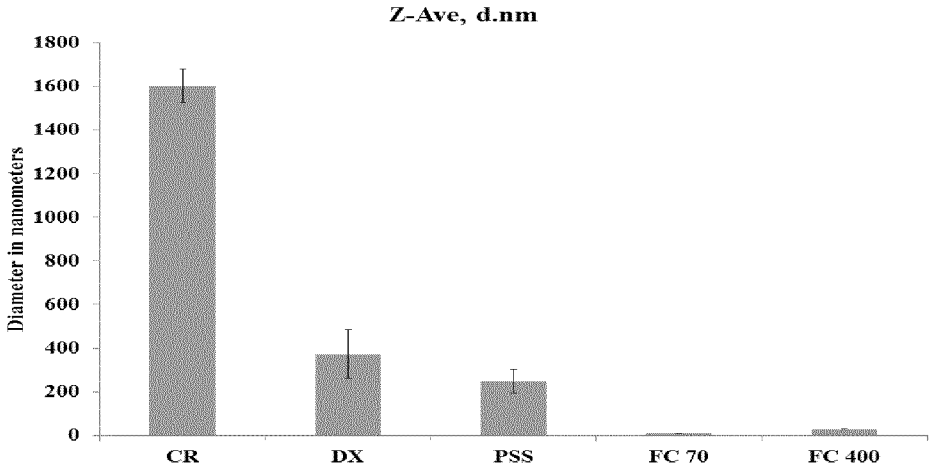
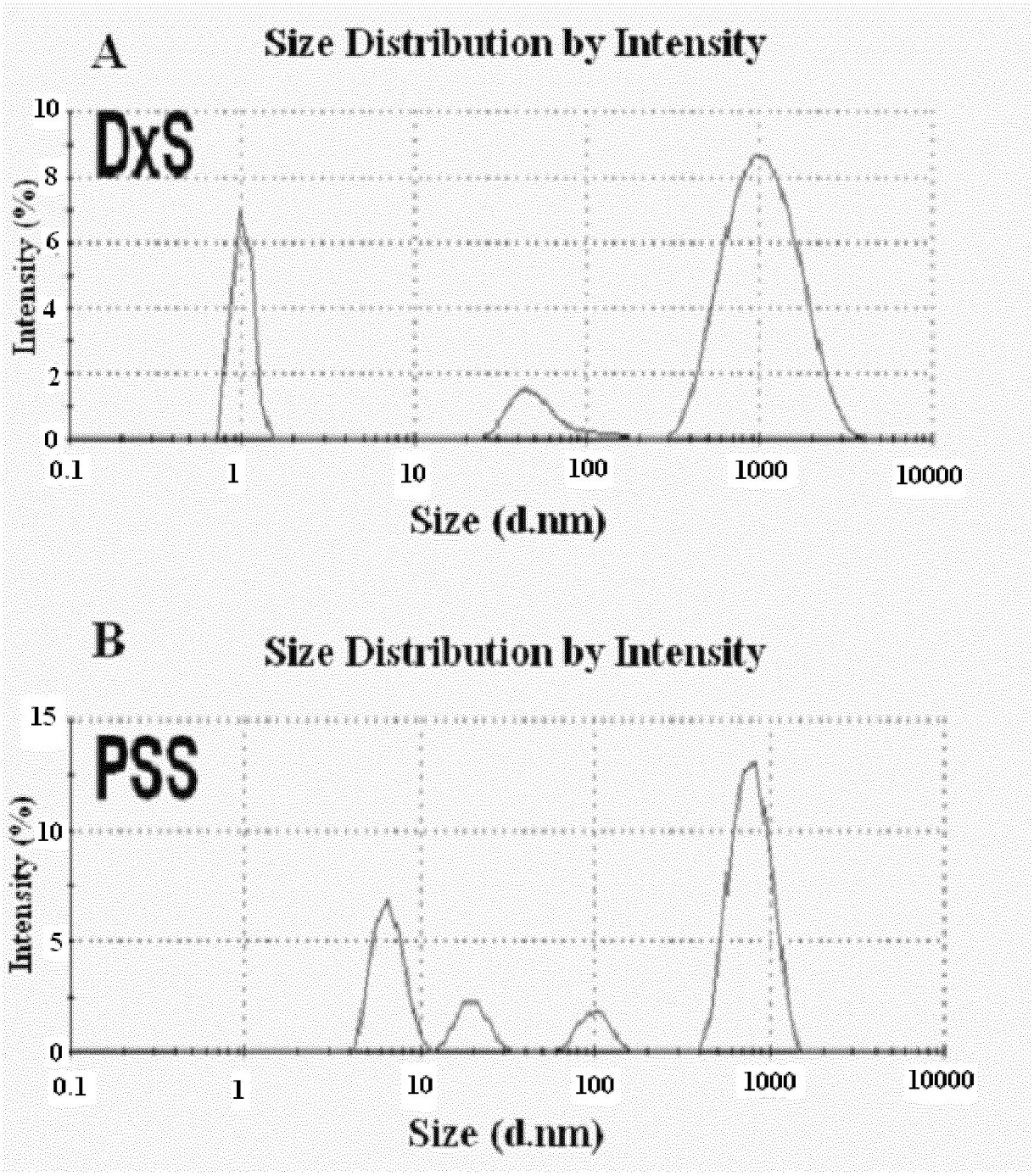
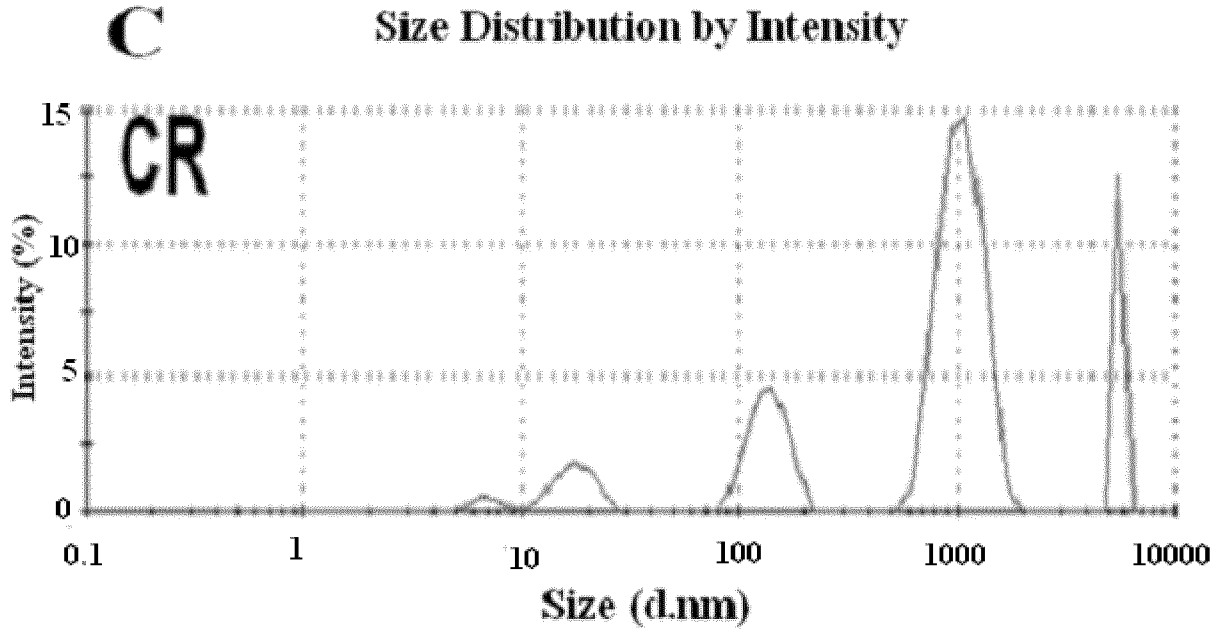


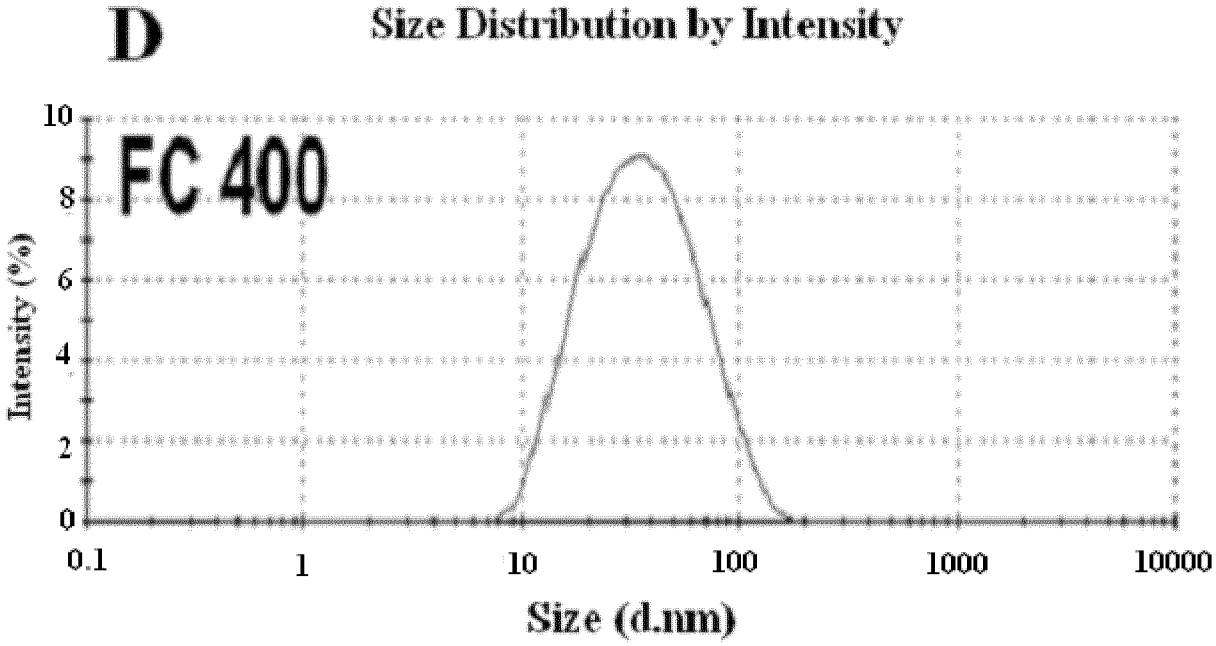
Figure 11A - E


A
Size Distribution by Intensity
DxS
Intensity (%)
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)
B
Size Distribution by Intensity
PSS
Intensity (%)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)

C


Size Distribution by Intensity
CR
Intensity (%)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)

D


Size Distribution by Intensity
FC 400
Intensity (%)
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)

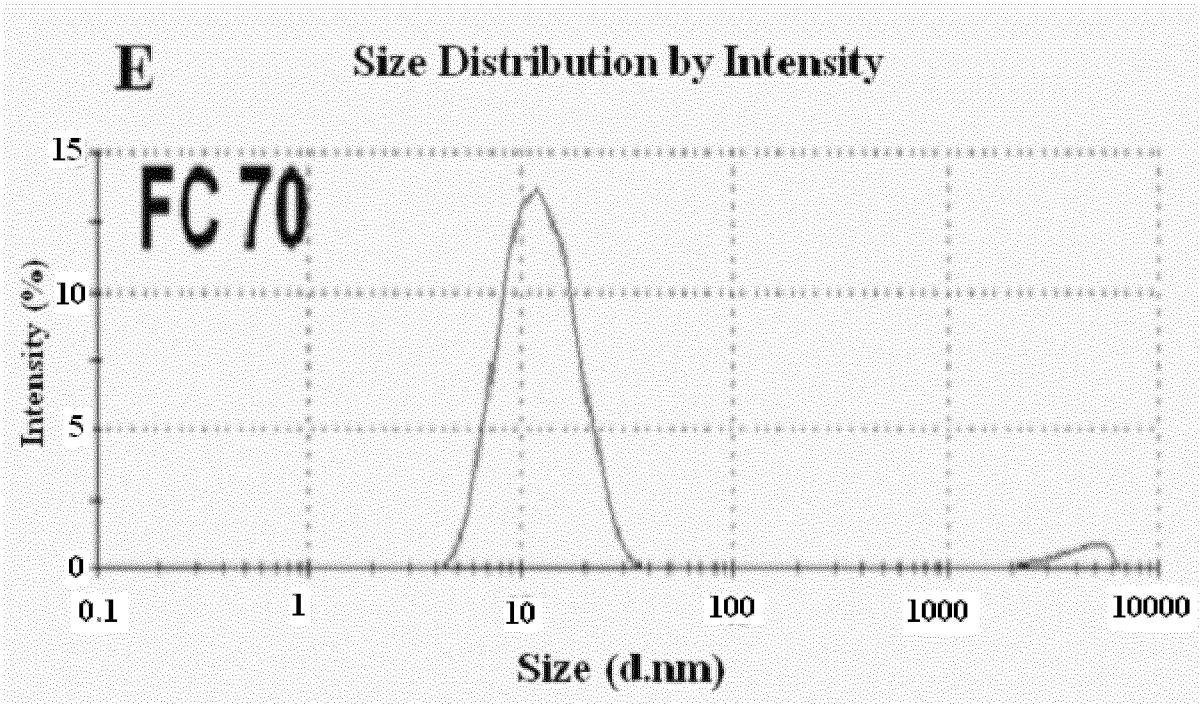
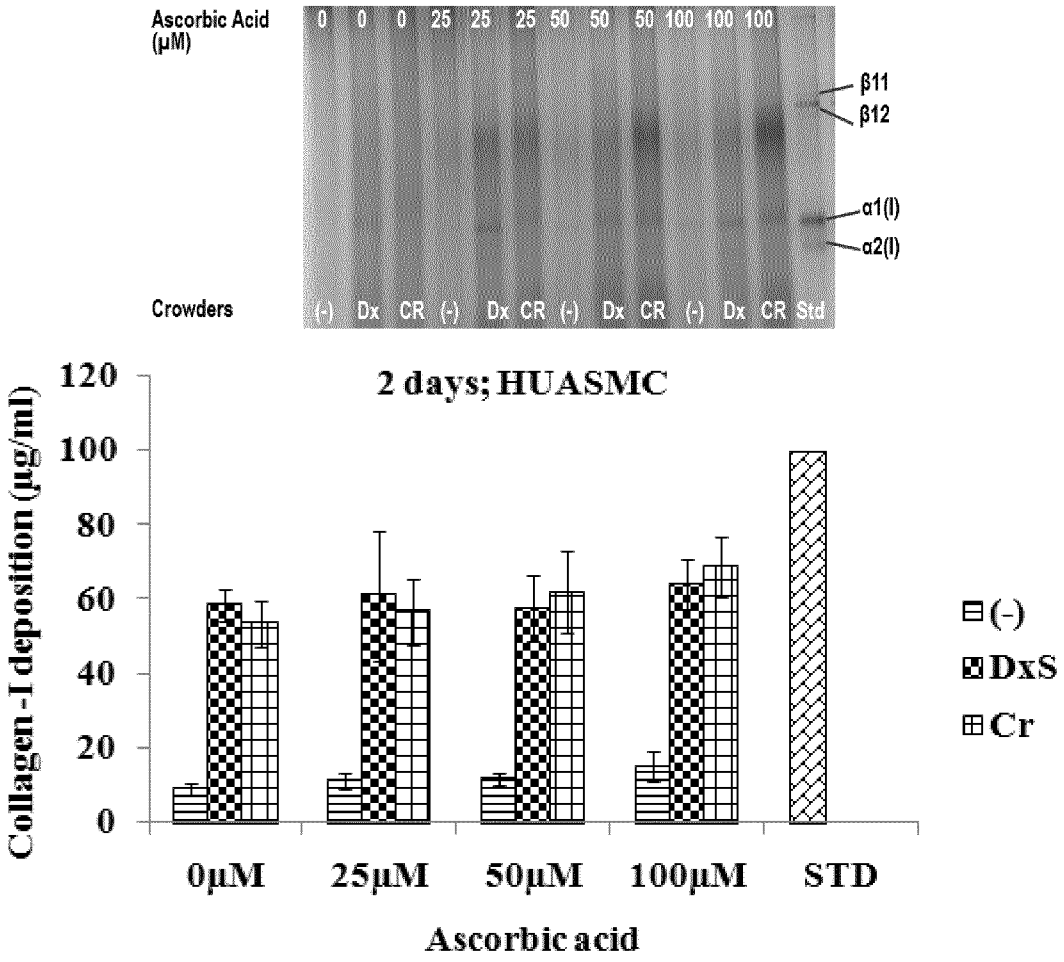

E
Size Distribution by Intensity
FC 70
Intensity (%)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)

Figure 12


Ascorbic Acid (μM)
0 0 0 25 25 25 50 50 50 100 100 100
β11
β12
α1(I)
α2(I)
Crowders
(-) Dx CR (-) Dx CR (-) Dx CR (-) Dx CR Std
2 days; HUASMC
Collagen-I deposition (μg/ml)
120
100
80
60
40
20
0
0μM
25μM
50μM
100μM
STD
Ascorbic acid
(-)
DxS
Cr

.

Figure 13:

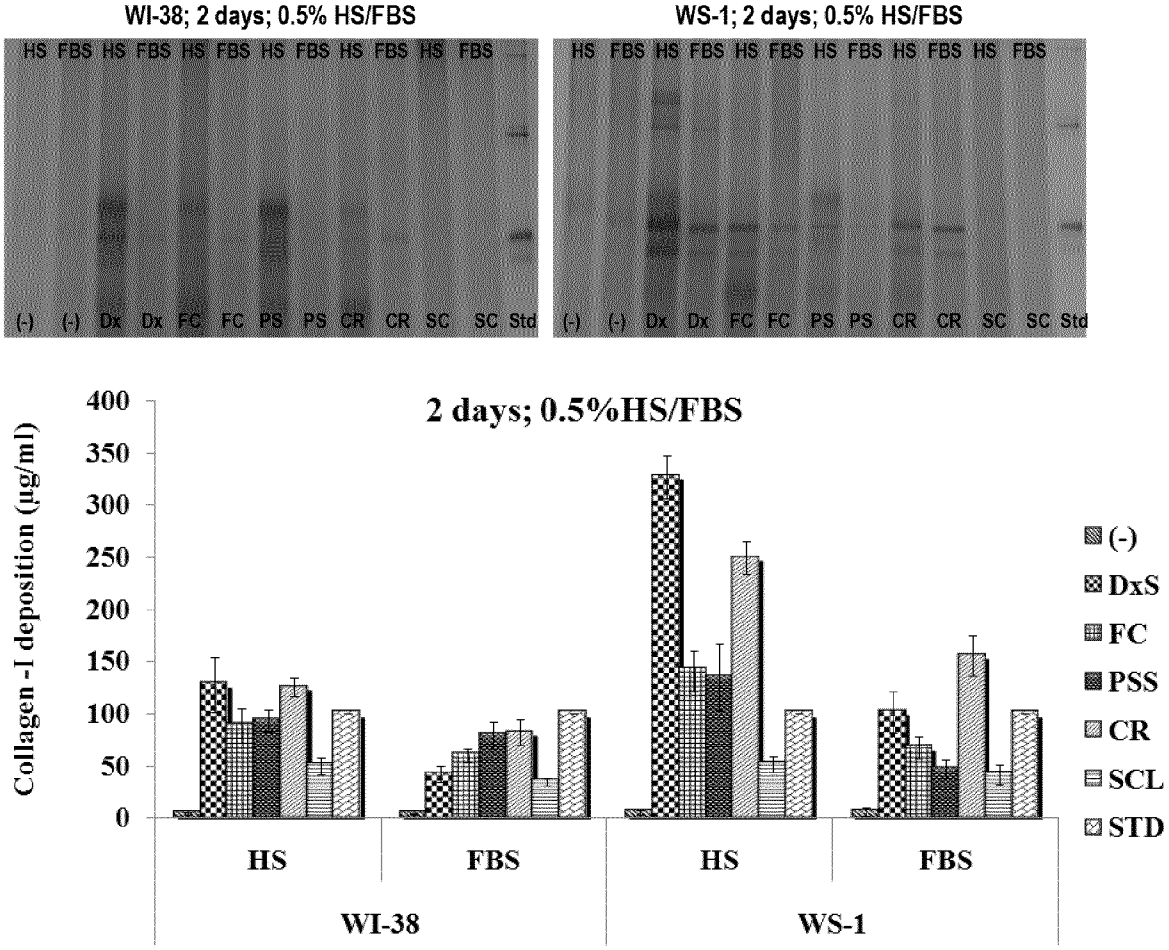
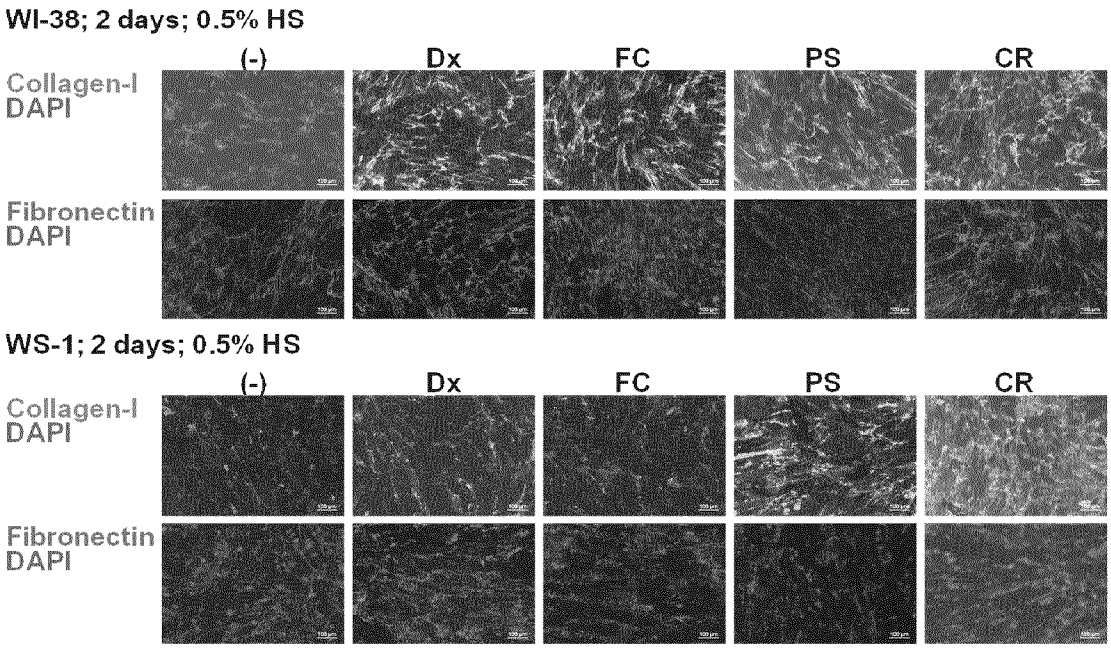


Figure 14:



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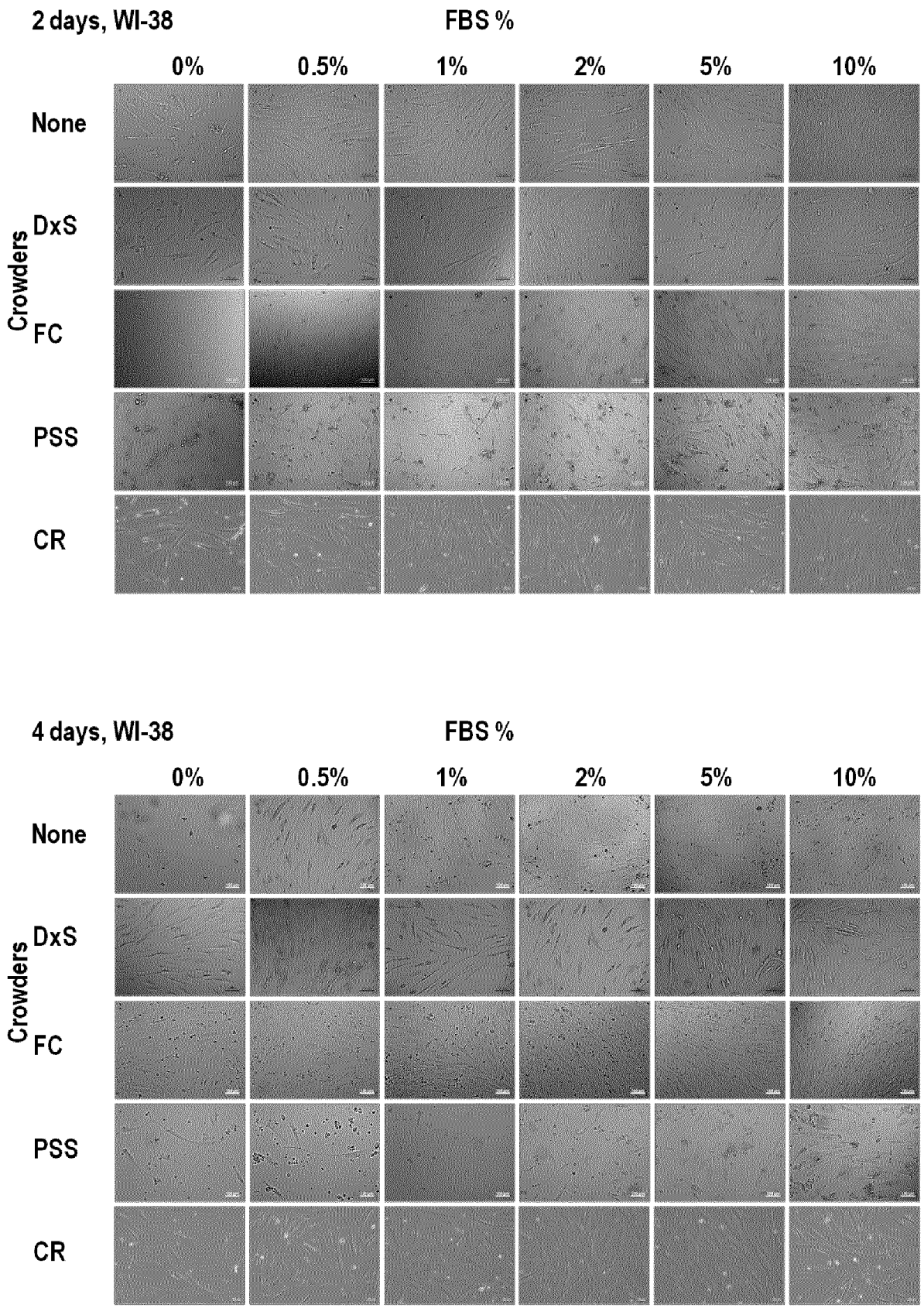
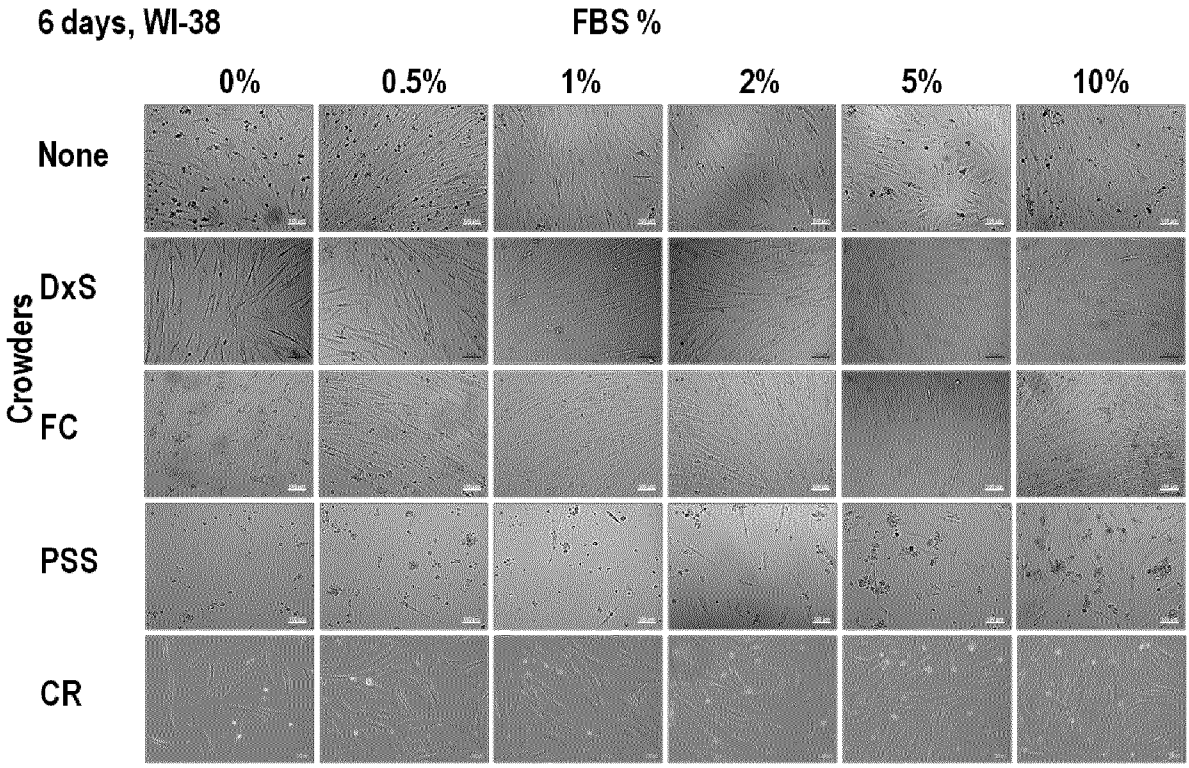
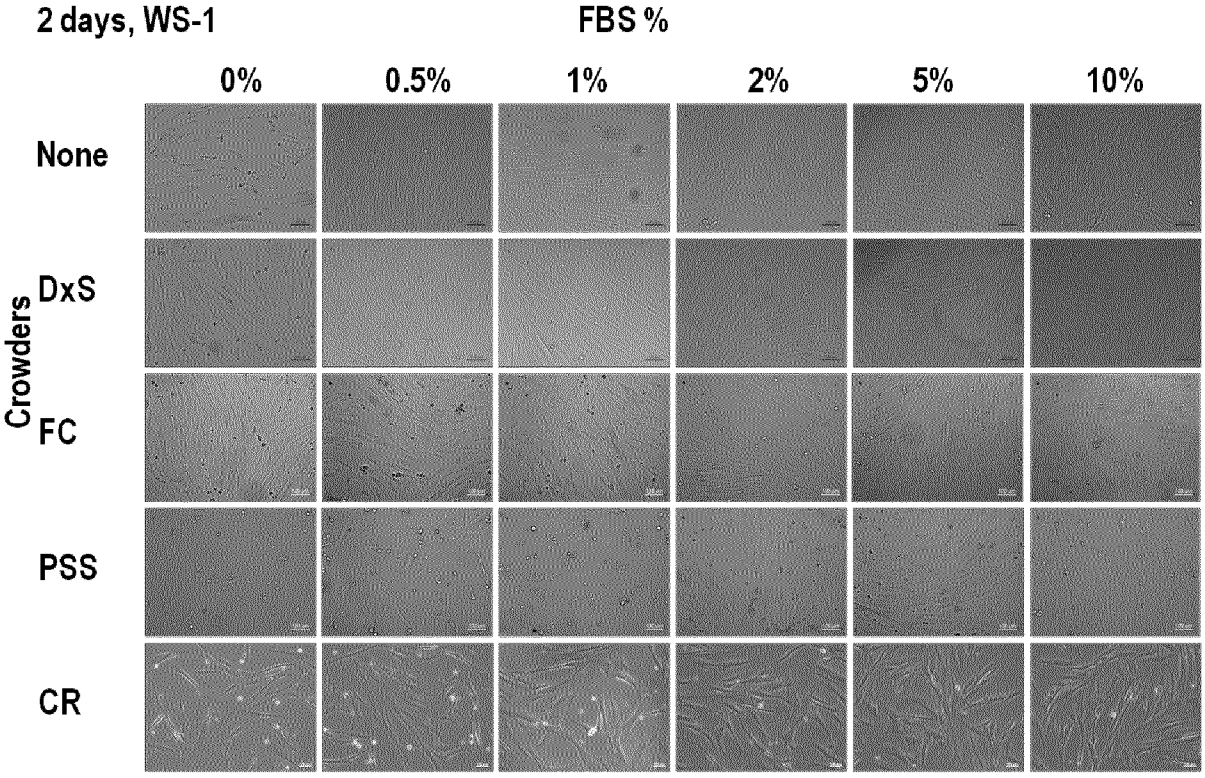
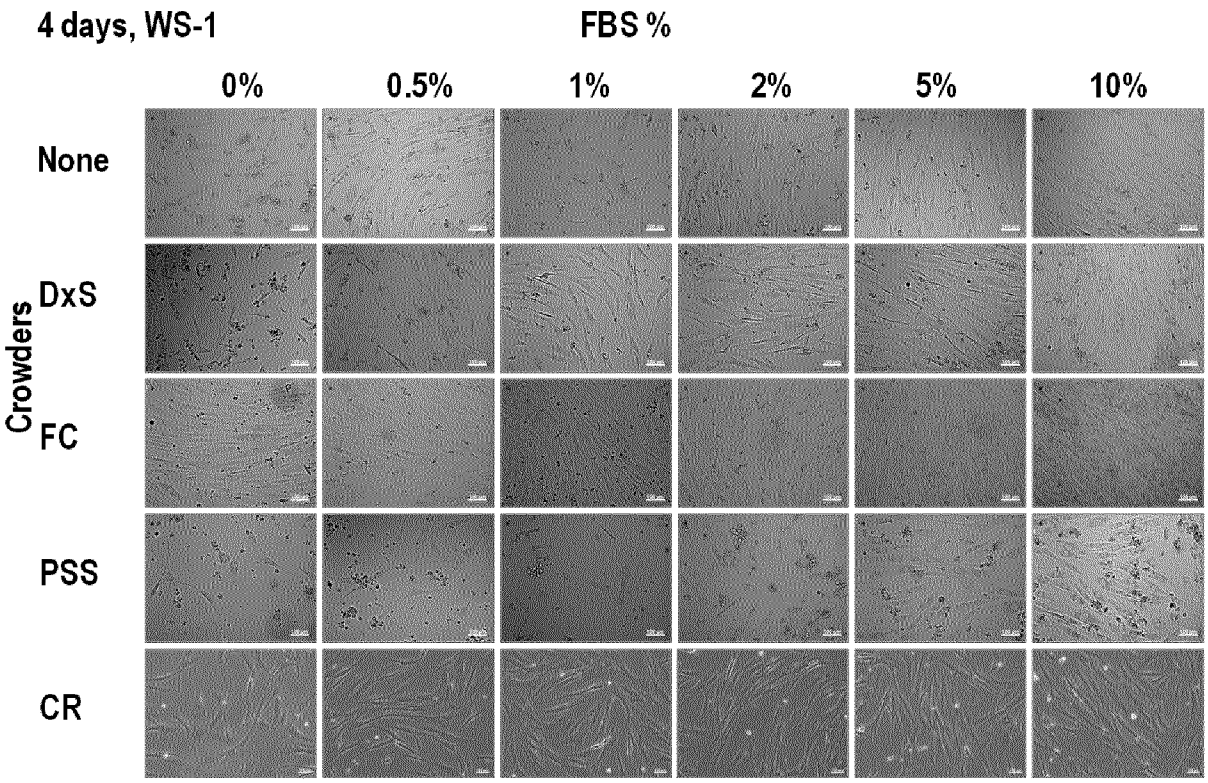
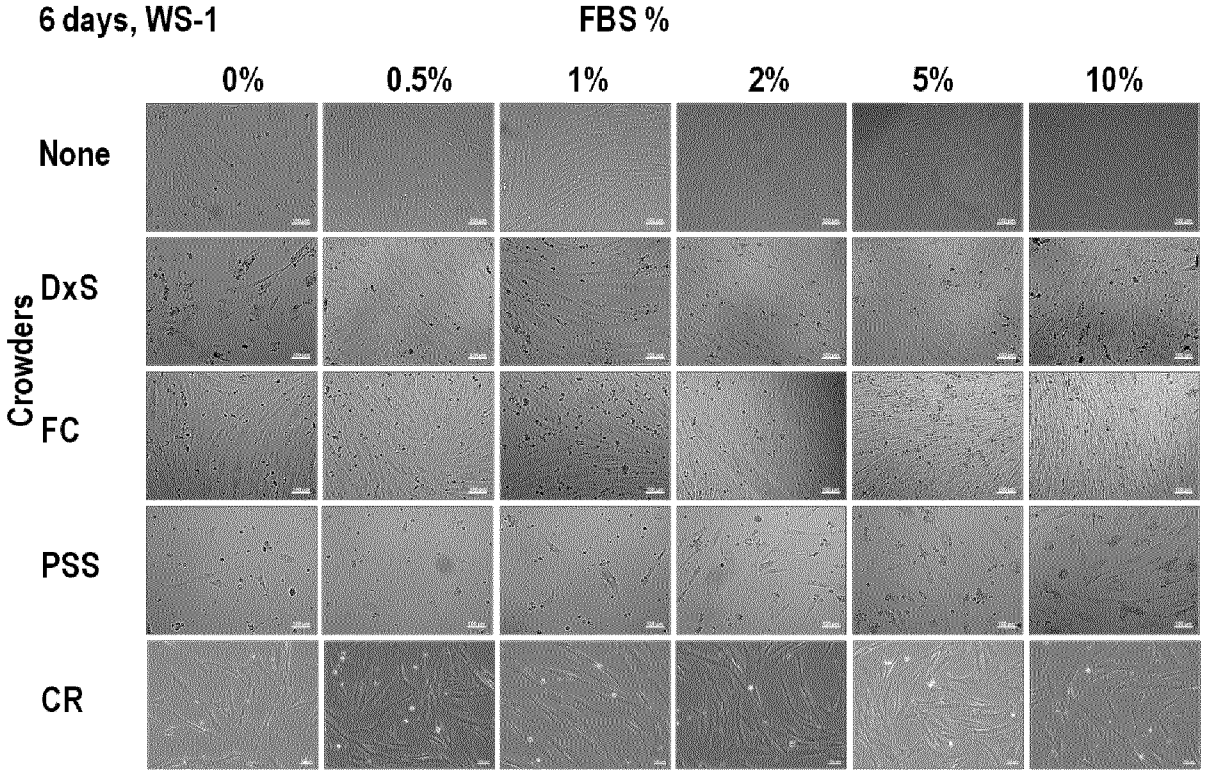

2 days, WI-38
FBS %
0%
0.5%
1%
2%
5%
10%
Crowders
None
DxS
FC
PSS
CR
4 days, WI-38
FBS %
0%
0.5%
1%
2%
5%
10%
Crowders
None
DxS
FC
PSS
CR


6 days, WI-38
FBS %
0%
0.5%
1%
2%
5%
10%
Crowders
None
DxS
FC
PSS
CR


2 days, WS-1
FBS %
0%
0.5%
1%
2%
5%
10%
Crowders
None
DxS
FC
PSS
CR


4 days, WS-1
FBS %
0%
0.5%
1%
2%
5%
10%
None
Crowders
DxS
FC
PSS
CR


6 days, WS-1
FBS %
0%
0.5%
1%
2%
5%
10%
None
Crowders
DxS
FC
PSS
CR

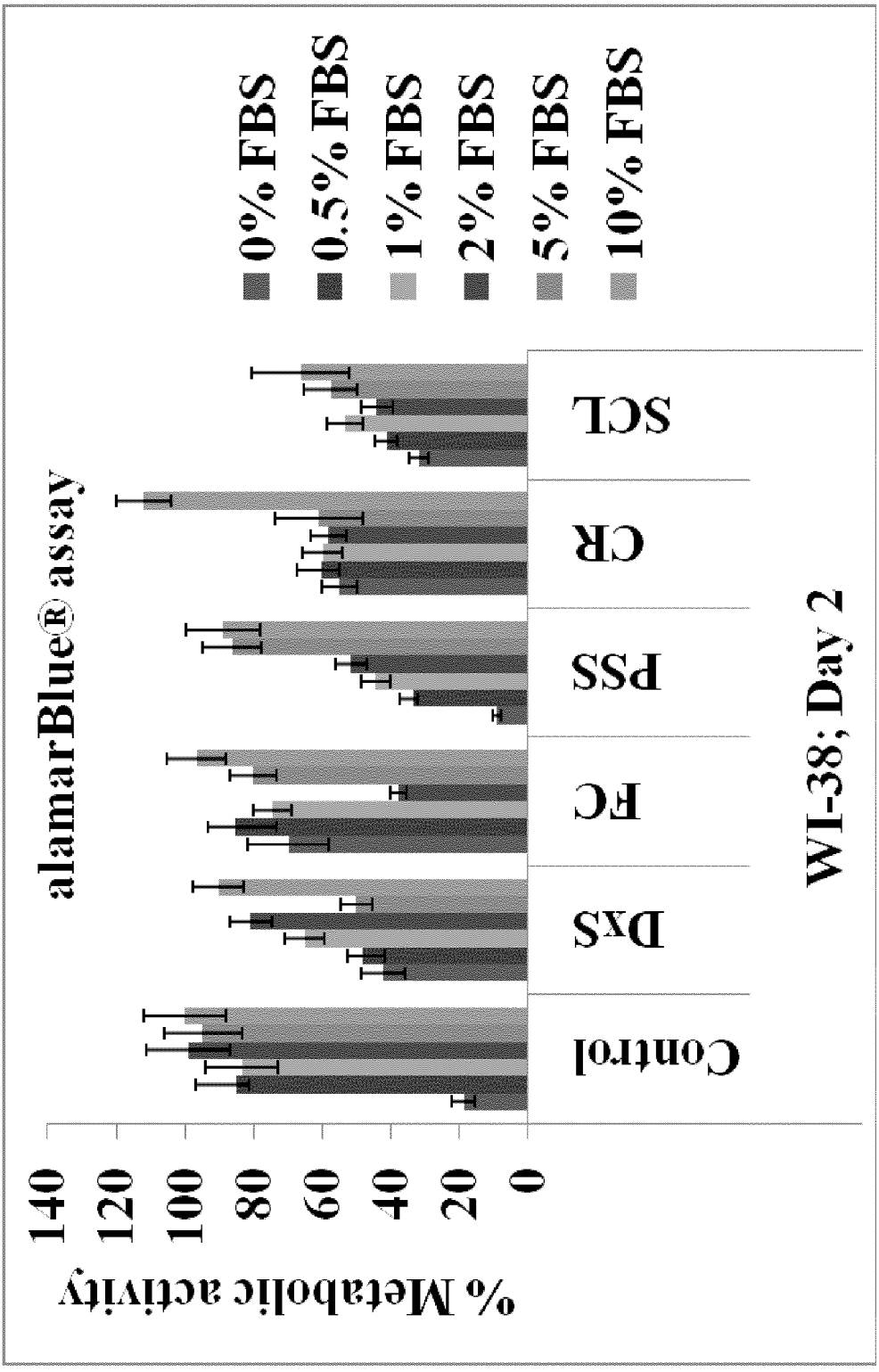


Figure 16

15 / 19

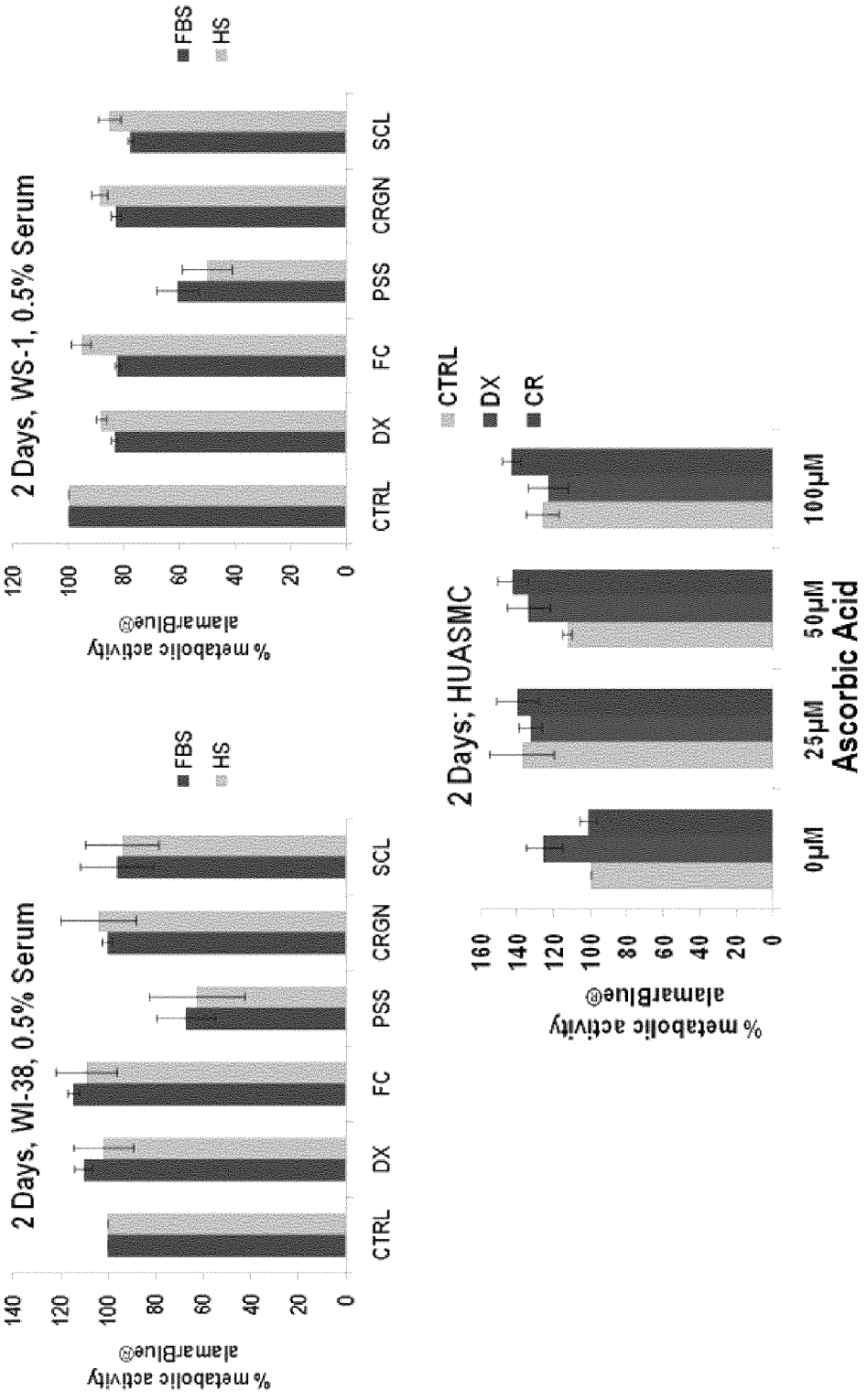

2 Days, WI-38, 0.5% Serum
% metabolic activity
alamarBlue®
FBS
HS
CTRL
DX
FC
PSS
CRGN
SCL
2 Days, WS-1, 0.5% Serum
2 Days; HUASMC
CTRL
DX
CR
0µM
25µM
50µM
100µM
Ascorbic Acid

Figure 17

17 / 19

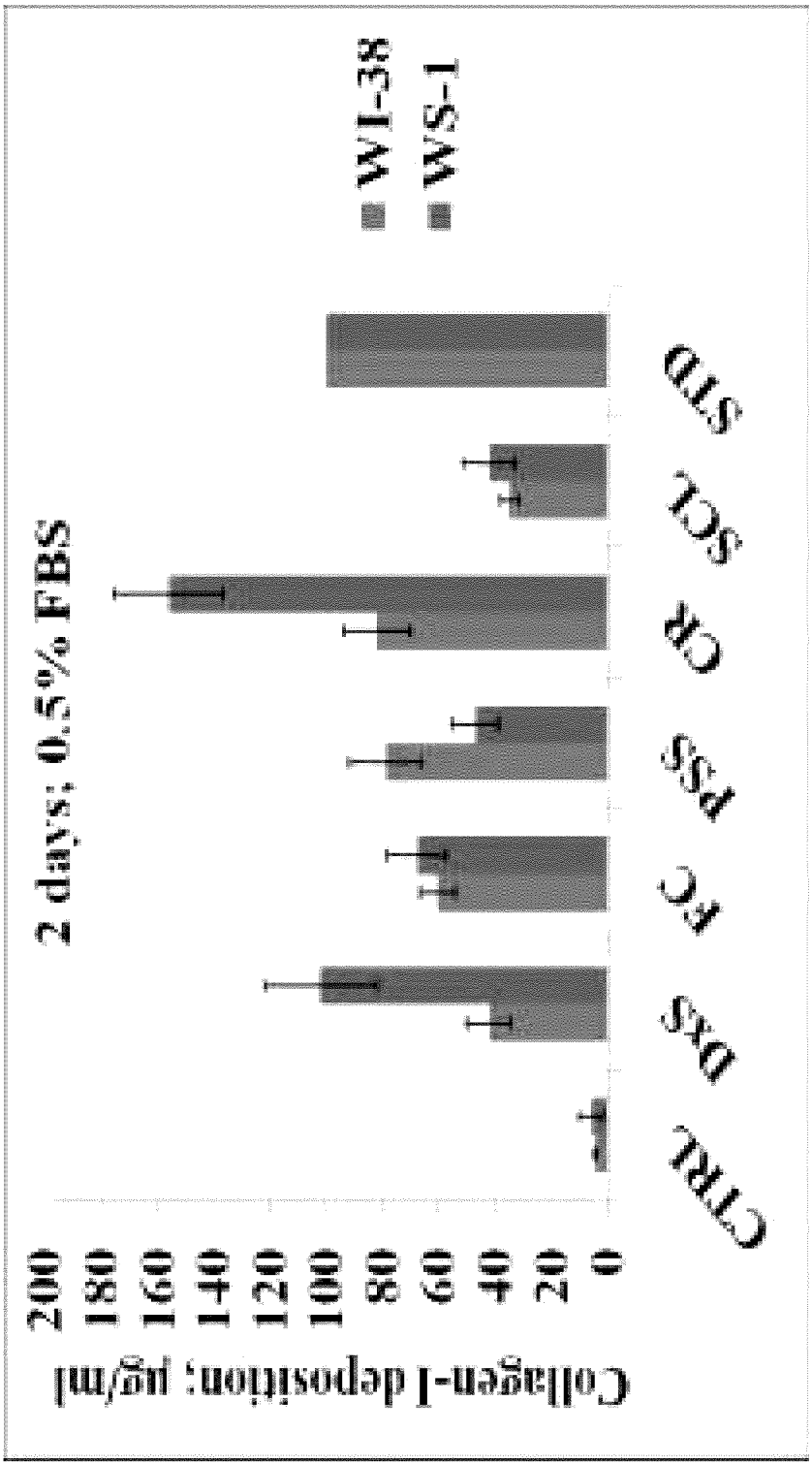


Figure 18

18 / 19

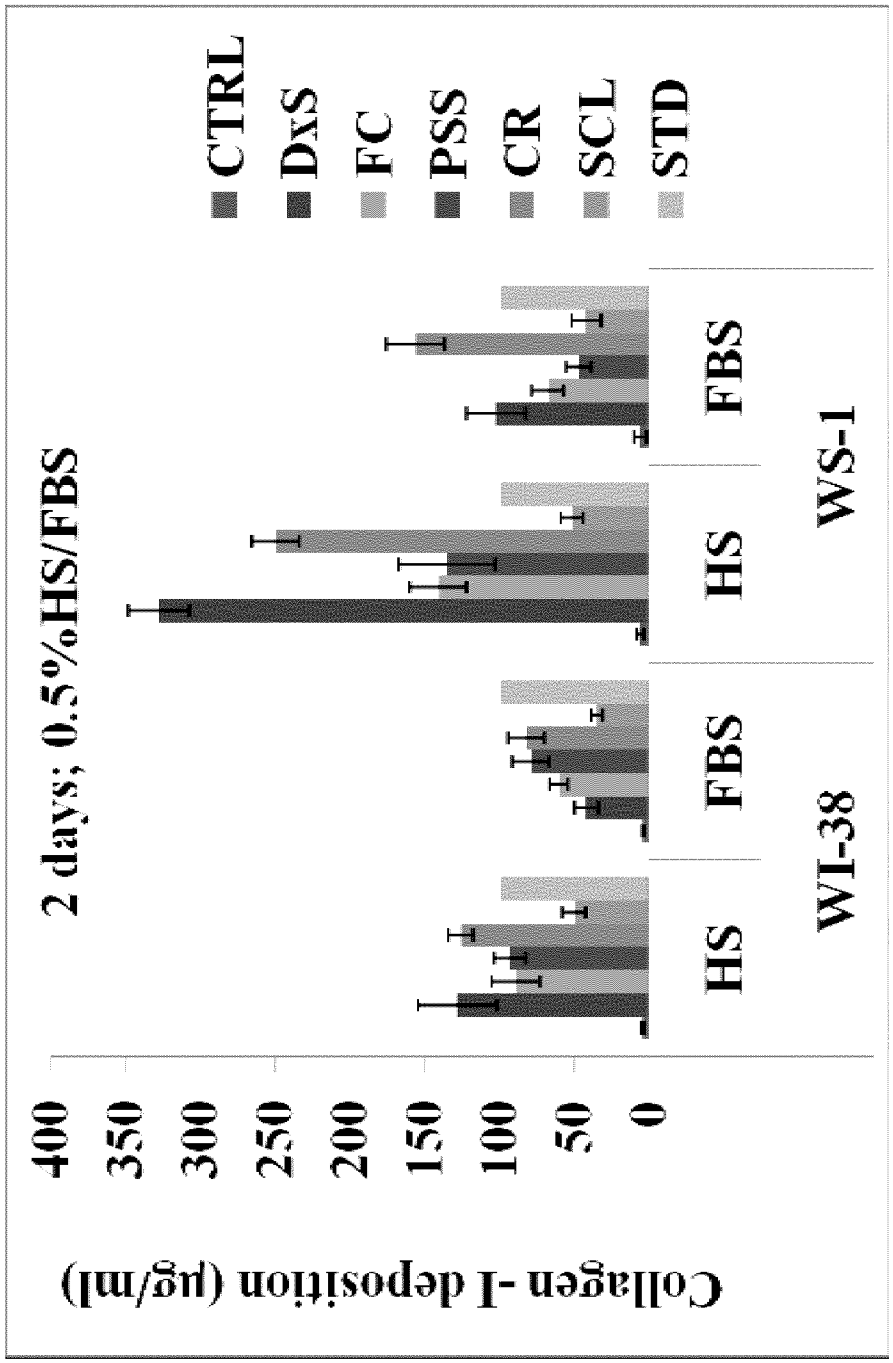


Figure 19

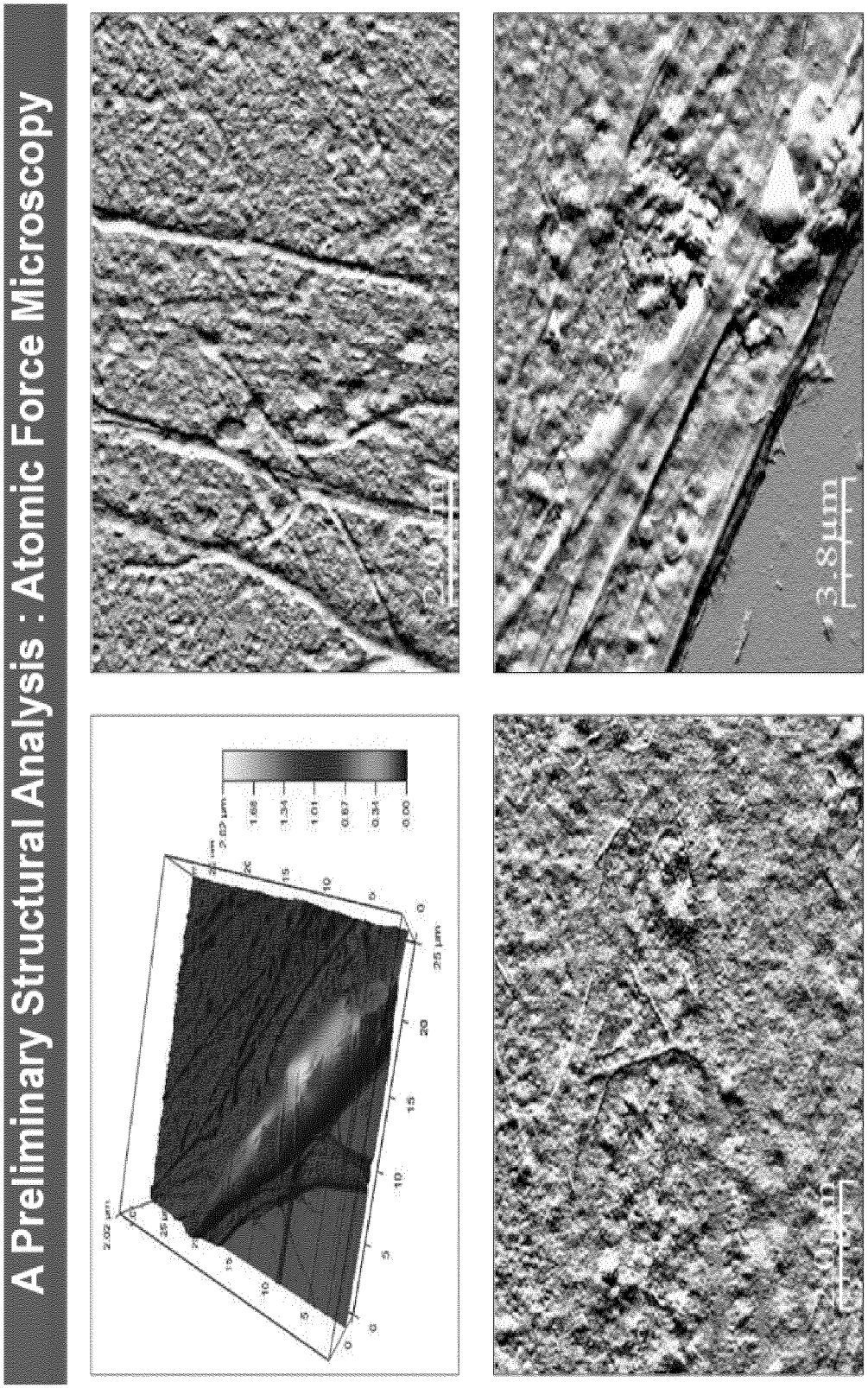

A Preliminary Structural Analysis : Atomic Force Microscopy
3.8µm
25 µm
2.02 µm
1.68
1.34
1.01
0.67
0.34
0.00

Figure 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/060945

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLARICE CHEN ET AL: "Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies", ADVANCED DRUG DELIVERY REVIEWS, vol. 63, no. 4-5, 1 April 2011 (2011-04-01), pages 277-290, XP055013967, ISSN: 0169-409X, DOI: 10.1016/j.addr.2011.03.003 cited in the application 3.Applications of MMC --- -/- ·	1,2, 5-12,15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2012	17/10/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mauhin, Viviane

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAREU ET AL: "Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged macromolecules: The biological relevance of the excluded volume effect" , FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol . 581 , no. 14, 31 May 2007 (2007-05-31) , pages 2709-2714, XP022100064, ISSN: 0014-5793 , D0I : 10. 1016/J . FEBSLET.2007 .05 .020 cited in the application the whole document	1,2, 5-12 ,15

X	RICKY R. LAREU ET AL: "In Vitro Enhancement of Collagen Matrix Formation and Crosslinking for Applications in Tissue Engineering: A Preliminary Study" , TISSUE ENGINEERING, vol . 13, no. 2, 1 February 2007 (2007-02-01) , pages 385-391 , XP055013970, ISSN: 1076-3279 , D0I : 10. 1089/ten .2006. 0224 cited in the application the whole document	1,2, 5-12 ,15

X	US 2002/028192 Al (DIMITRIJEVICH S DAN [US] ET AL) 7 March 2002 (2002-03-07)	10-12
A	the whole document	1-9 ,15

X	W0 2006/097701 A2 (INTERCYTEX LTD [GB] ; KEMP PAUL [GB] ; SHERING DAVID [GB] ; JOHNSON PENNY) 21 September 2006 (2006-09-21)	10-12
A	the whole document	1-9 ,15

X	US 2005/089512 Al (SCHLOTMANN KORDULA [DE] ET AL) 28 April 2005 (2005-04-28)	10, 11
A	the whole document	1-9

A	DHAWAL SHAH ET AL: "Effects of polydisperse crowders on aggregation reactions: A molecular thermodynamic analysis", THE JOURNAL OF CHEMICAL PHYSICS, vol . 134, no. 6, 9 February 2011 (2011-02-09) , XP055033854, D0I : 10. 1063/1 .3549906] the whole document	1-15

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEN HAI-MIN ET AL: "1ambda-Carrageenan oligosacchari des elicit reacti ve oxygen speci es producti on resul ting in mi tochondri al-dependent apoptosi s in human umbi l i cal vei n endothel i al cel l s", INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE, vol . 24, no. 6, December 2009 (2009-12) , pages 801-806, XP002665126, ISSN: 1107-3756 the whol e document ----	1-9
A	R. C. PEREI RA ET AL: "Novel i njectabl e gel (system) as a vehi cl e for human arti cul ar chondrocytes i n carti l age t issue regenerati on", JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE, vol . 3, no. 2, 1 February 2009 (2009-02-01) , pages 97-106, XP055013974, ISSN: 1932-6254, D0I: 10. 1002/term. 145 the whol e document ----	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/06Q945

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

4(completely) ; 1-3 , 5-12, 15(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 4(completely) ; 1-3, 5-12, 15(partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of a large poly-dispersed macromolecule in its general definition, preferably carrageenan; Product obtained by the method. Culture medium containing a large molecular crowder in its general definition.

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2. claims: 1-3, 5-12, 15(all partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of dextrans ; Product obtained by the method.

   ---

3. claims: 1-3, 5-12, 15(all partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of dextran sulphate; Product obtained by the method.

   ---

4. claims: 1-3, 5-12, 15(all partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of Ficoll; Product obtained by the method.

   ---

5. claims: 1-3, 5-12, 15(all partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of bovine serum albumin; Product obtained by the method.

   ---

6. claims: 1-3, 5-11, 15(all partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of sodium algerate; Product obtained by the method.

   ---

7. claims: 1-3, 5-12, 15(all partially)

   Method for the production of a tissue substitute comprising culturing cells in the presence of polyethylene glycol ; Product obtained by the method.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

8. claims: 1-3, 5-12, 15(all partially)

Method for the production of a tissue substitute comprising culturing cells in the presence of sepharose-CL; Product obtained by the method.

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9. claims: 1-3, 5-12, 15(all partially)

Method for the production of a tissue substitute comprising culturing cells in the presence of polysodium-l-styrene sulphonate; Product obtained by the method.

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10. claim: 13

Method for increasing the speed of an in vitro assay

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11. claim: 14

Diagnostic

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/060945

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002028192 A1	07-03-2002	NONE	
WO 2006097701 A2	21-09-2006	AT 417922 T	15-01-2009
		AU 2006224355 A1	21-09-2006
		AU 2011200128 A1	03-02-2011
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US 2005089512 A1	28-04-2005	NONE	